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(54) Title: OLIGONUCLEOTIDES COMPRISING INTERCALATOR PSEUDONUCLEOTIDE(S) FOR DETECTION OF NUCLEIC ACIDS AND MUTANTS HEREOF

(57) Abstract: The present invention relates to oligonucleotides or oligonucleotide analogues comprising at least two intercalator pseudonucleotides. Intercalator pseudonucleotides are capable of being incorporated into the backbone of a nucleic acid analogue and comprise an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. Furthermore, the invention relates to methods of detecting nucleic acids or nucleic acid analogues comprising a specific target sequence, methods of differentiating between a nucleic acid or nucleic acid analogue comprising a specific target sequence and a nucleic acid comprising a mutant sequence which differs from the target sequence by at least one nucleobase, and methods for detecting a target sequence and/or a mutant sequence, which offer from the target sequence by at least one nucleobase. In preferred embodiments of the invention said methods involve the use of oligonucleotides or oligonucleotide analogues comprising at least two intercalator pseudonucleotides.



WO 03/052134 A2

Oligonucleotides comprising intercalator pseudonucleotide(s) for detection of nucleic acids and mutants hereof

Field of the invention

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The present invention relates to the field of detecting specific nucleic acid sequences; such as mutations, in particular single point mutations. Furthermore, the invention relates to the field of pseudonucleotides comprising intercalators. In particular the invention relates to detecting mutations using oligonucleotides or oligonucleotide analogues comprising intercalators.

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Background of the invention

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Nucleic acids, such as DNA, RNA as well as a number of nucleic acid analogues such as [Den store liste fra patent 1 skal indsættes- søg og erstat gennem dokumentet] PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA are capable of specifically hybridising to their complementary strands. This specific recognition may be utilised to detect the presence of specific nucleic acid sequences for example for diagnostic purposes. It is possible to detect very small differences in nucleic acid sequence, even a single nucleotide substitution.

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Many of the available assays rely on the detection of a specific nucleic acid by a complementary detectable probe. It is often required to separate unbound probe from bound probe in order to detect the specific nucleic acid, and this usually requires several time-consuming separation steps.

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DNA diagnostics is one of the fastest growing research areas, and now with the draft of the human genome map available aiming for the full sequence in detail, the interest in the field is expected to expand even further. A map of 1.42 million single

nucleotide polymorphisms (SNPs) has been described and it has been estimated that at least 60,000 SNPs fall within the human exons. The search for sequences that only differ in one or two nucleobases, creates the need for tools for detecting nucleic acid sequences with high performance, cost.that are fast and simple to conduct, and are cost effective.

Interestingly, the genomic DNA sequence differs in average in about one out of 1000 nucleotides between two human beings. Accordingly, specific DNA sequences may be useful for determining the identity of an individual. Furthermore, mutations may be indicative of predisposition to clinical conditions.

A classic example in genetic diseases is Sickel cell anaemia, a genetic defect caused by a change of a single base in a single gene: the beta-globin gene (GAG is changed to GTG at Codon 6).

Different techniques to detect mutations in nucleic acid sequences for example includes single-stranded conformational analysis, denaturing gradient gel electrophoresis, heteroduplex analysis, chemical mismatch cleavage and direct sequencing. A review of these techniques has been given by Grompe. Among the variety of techniques also fluorescence based methods are available.

Pyrene is an excimer-forming molecule, which has been incorporated into oligodeoxynucleotides (ODNs) by several groups. Ebata et al. incorporated a pyrene-modified nucleotide in the 5' end of one ODN and a pyrene-modified nucleotide into the 3' end of another. By hybridising to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated. Paris et al. published a similar system where the utility of the system to detect mismatches was also explored. The ability of this system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridise in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.

In the prior art synthetic nucleotide like molecules comprising intercalators have been described:

US 5,446,578 describes synthetic nucleotide like molecules comprising fluorescent molecules, which shows a change in spectra with concentration, for example pyrene. In particular, the document describes nucleic acids derivatised with such fluorescent molecule on the phosphate of a nucleic acid backbone or nucleic acids comprising an acyclic backbone monomer unit consisting of 5 atoms between two phosphates of the nucleic acid backbone, coupled to such a fluorescent molecule. The document states that the fluorescent molecules should be positioned at the exterior of a nucleic acid helix so that they are not capable of intercalating with nucleobases of a nucleic acid. Furthermore, it is explained that the fluorescence of the fluorescent molecule increases upon hybridisation and that a cationic surfactant must be present to achieve this effect.

Yamana et al., 1999, describes an oligonucleotide containing a 2'-O-(1-pyrenylmethyl)uridine at the center position. Said oligonucleotide has higher affinity for DNA and lower affinity for RNA compared to an unmodified oligonucleotide. Upon hybridisation monomer and excimer fluorescence is enhanced.

Yamana et al., 1997, describes a phosphoramidite coupled to pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidite may be incorporated into a nucleic acid as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Upon hybridisation, excimer fluorescence is greatly enhanced and nucleic acids into which said phosphoramidites have been incorporated retain normal binding affinity for DNA.

Korshun et al., 1999, describes a phosphoramidite coupled to a pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidite may be incorporated into a nucleic acid as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Furthermore the document describes oligonucleotides into which said phosphoramidites have been incorporated and it is described that the oligonucleotides have

higher affinity for DNA, than an unmodified oligonucleotide. It is mentioned that close coplanar mutual approach of two pyrene residues located in the neighboring positions of a modified oligonucleotide chain is strongly inhibited because of the small length of the linker. Excimer fluorescence increases upon hybridisation, however oligonucleotides comprising 5 such pyrene pseudonucleotides at the end of the oligonucleotide exhibit high excimer fluorescence when unhybridised as well.

US 5,414,077 describes pseudonucleotides, which may comprise an intercalator such as an acridine or anthraquinone. The pseudonucleotide comprises an achiral or a single enantiomer organic backbone, such as diethanolamine. The pseudonucleotides may be incorporated at any desired position within an oligonucleotide. Such oligonucleotides in general have higher affinity for complementary nucleotides, in particular when the pseudonucleotides are inserted at the end. The document does not describe fluorescence data.

US 6,031,091 describes pseudonucleotides which may be incorporated at any position in an oligonucleotide. In particular the document describes acyclic phosphor containing backbones and it is mentioned that the pseudonucleotides may comprise an intercalator. Specific pseudonucleotides described in the document comprises very long linkers connecting polyaromates to the nucleic acid backbone and accordingly said pseudonucleotides.

EP 0 916 737 A2 describes polynucleotides derivatised with for example intercalating compounds. The intercalating compounds should preferably be separated by approx. 10 nucleotides. The polynucleotide may be derivatised on the phosphate, the sugar or the nucleobase moiety. In particular, they may be derivatised on the nucleobase by a 7 or a 11 atoms long linker coupled to a polyaromate in a manner that does not interfere with Watson-Crick base pairing. It is stated that fluorescence intensity is enhanced by intercalation.

Strässler et al., 1999, describes pseudonucleoside comprising a fluorescent molecule for example pyrene instead of a nucleobase.

WO 97/43298 describes nucleoside analogues comprising a polyaromatic hydrocarbon for example pyrene attached to the 1' position of ribose or deoxyribose as well as phosphoramidite derivatives of said polyaromatic hydrocarbons.

5 **US 5,175,273** describes nucleotide derivatives comprising nucleobases fused to planar polycyclic aromatic compounds. Oligonucleotide comprising said nucleotide derivatives have increased affinity for DNA and fluorescence is decreased by hybridization.

10 **US 6,153,737** describes nucleotide derivatised on the 2' position with pyrene or another intercalator.

Ebata et al., 1995 describes incorporation of a pyrene-modified nucleotide in the 5' end of a DNA oligonucleotide and a pyrene-modified nucleotide into the 3' end of
15 another. By hybridising to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated.

Paris et al., 1998 described a system similar to the one disclosed by Ebata et al.
20 wherein the system may be utilised to detect mismatches. However the ability of the system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridise in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.
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Summary of the invention

This application claims benefit under § 119(e) to U.S. provisional patent application
30 Serial No. 60/365,545 filed 20 March 2002, which is hereby incorporated by reference in its entirety.

All patent and non-patent references cited in the application, or in the present application, are also hereby incorporated by reference in their entirety.

5 There is a need for materials and methods which allow for detection of single nucleotide polymorphisms (SNPs) and other mutations involving only a few base pairs, and which are fast, easy to perform, reliable and cheap.

Surprisingly, the inventors have discovered that a number of oligonucleotides comprising at least one intercalator fulfil the above criteria.

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Hence, it is a first objective of the present invention to provide oligonucleotide analogues comprising at least two intercalator pseudonucleotides of the general structure

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$$X-Y-Q$$

wherein

20

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

25

Y is a linker moiety linking said backbone monomer unit and said intercalator,

and wherein the at least two intercalator pseudonucleotides are separated by n nucleotides, wherein n is selected from the group consisting of integers in the range from 1 to 10.

30

Preferably every two intercalator pseudonucleotides are separated by at least 1 nucleotide.

35

It is second objective of the present invention to provide methods of detecting a nucleic acid or nucleic acid analogue comprising a specific target sequence, which

may differ from any other sequences by at least one nucleobase, wherein the method comprises the steps of

- 5 a) providing a mixture of nucleic acids and/ or nucleic acid analogues, which is desirable to test for the mutation; and
- b) providing an oligonucleotide analogue comprising at least one intercalator pseudonucleotide capable of hybridising with said specific sequence; and
- 10 c) incubating the oligonucleotide analogue with the mixture comprising nucleic acids or nucleic acid analogues under conditions allowing for hybridisation; andd) washing away sequences that have less affinity to said oligonucleotide analogue than the target sequence; ande) determining the presence or absence the target sequence.

15 It is a third objective of the present invention to provide methods of differentiating between a nucleic acid or nucleic acid analogue comprising a specific target sequence and a nucleic acid comprising a mutant sequence which differs from the target sequence by at least one nucleobase, wherein the method comprises the steps of

- 20 a) providing a mixture of nucleic acids or nucleic acid analogues, which is desirable to test for the presence of a target sequence or a mutant sequence; and
- b) providing an oligonucleotide analogue comprising at least one intercalator pseudonucleotide; and
- 25 c) incubating said oligonucleotide analogue with the mixture comprising nucleic acids or nucleic acid analogues under conditions allowing for hybridization; and
- d) determining the presence or absence of said target sequence and/or the presence of the mutant sequence.

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Legends to figures

Figure 1 illustrates the synthesis of an intercalator pseudonucleotide, a phosphoramidite as depicted in 5.

Figure 2 illustrates a structural calculation of the self-complementary DNA duplex with the sequence 5'-XCGCGCG-3' done in "MacroModel", X = the pyrene module. The pyrene moiety is co-axial stacked with the underlying base pair.

5

Figure 3 illustrates a slice of the calculated structure of the duplex 5'-AGCTTGCCTTGAG-3' + 5'-CTCAAGXCAACCT-3', X = 5. The pyrene makes co-axial stacking with both the upper and lower neighboring nucleobases of the opposite strand.

10

Figure 4 illustrates the calculated structure of a 12/13-mer duplex with the sequence 5'-AGCTTGCCTTGAG-3' + 5'-CTCAAGXCAACCT-3', X = 5 (figure 1). The pyrene moiety is able to interact with both the upper and lower neighboring nucleobases of the opposite strand. The distance between the nucleobases and the pyrene moiety is shown to the right.

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Figure 5 illustrates fluorescent measurements of a 13-mer, mono pyrene inserted ssDNA (★); its duplex with complementary, 12-mer RNA () and its duplex with complementary, 12-mer DNA (◆). The sequences are the same as those shown in Table 3.

20

Figure 6 illustrates fluorescent measurements of a 14-mer ssDNA with two pyrene insertions separated by one nucleotide (★); its duplex with complementary, 12-mer RNA () and its duplex with complementary, 12-mer DNA (◆). The sequences are the same as those shown in Table 3.

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Figure 7 illustrates a procedure to prepare a sample for RT-PCR

Figure 8 illustrates a procedure to prepare a sample for RT-PCR

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Figure 9 illustrates a procedure to prepare a sample for RT-PCR

Figure 10 illustrates a procedure to prepare sequence specific DNA

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Figure 11 illustrates a procedure to prepare a sequence specific DNA

Figure 12 illustrates a method to detect sequence specific DNA using a chip

Figure 13 illustrates different kinds of oligonucleotides that may be useful as probes
5 on a chip

Figure 14 illustrates PCR quantification.

10 **Figure 15 illustrates transcription blockage using a pair of oligonucleotides according to the invention indicated as A and B, respectively.**

Figure 16: Nuclease resistance of two oligonucleotides whereof one comprises intercalating pseudonucleotides (INA oligo) and the duplex of said two oligonucleotides.
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Figure 17: Secondary structure of the hairpin forming probe I. In this conformation the monomer and excimer fluorescence is quenched.

20 **Figure 18:** Secondary structure of probe I when hybridised to a target sequence. When hybridized to a target sequence, the excimer complex is free to be formed and hence excimer fluorescence can be observed. The monomer fluorescence is also increased.

25 **Figure 19** SYBR green II stained INA oligos, visualized on an ArrayWorx scanner.

Figure 20: illustrates a test of oligo binding on Asper SAL slides.

Figure 21: Exciplex fluorescence between molecules X and Y when placed as next-nearest neighbours (Sequence I)
30

Figure 22: Exciplex fluorescence between molecules X and Y when placed as neighbours (Sequence II)

35 **Figure 23:** Exciplex fluorescence between molecules Y and Z when placed as neighbours

Figure 24: illustrates EtBr staining

Figure 25: Sequence of the employed double-stranded target oligo, the attacking IOs and the complimentary pairing IOs. Y denote intercalating units.

Figure 26: IOs spontaneously bind target DNA.

Reactions were carried out in 20 μ l volumes containing 126 nM IOs with or without 20 nM target DNA, for 1 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

Figure 27: IO-DNA complex formation requires sequence complementarity.

Reactions were carried out in 15 μ l volumes containing the indicated concentrations of IOs with or without 20nM target DNA (single or double stranded), for 2 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

Figure 28: IO pairing in spontaneous target binding.

Reactions were carried out in 15 μ l volumes containing 20nM target DNA and the indicated concentrations of pre-annealed P32-labelled IOs for 2 h at 37°C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

Figure 29: Pairing does not affect the efficiency of spontaneous binding.

Reactions were carried out in 15 μ l volumes containing 20nM target DNA and increasing amounts of IOs (40-80-160 nM) as indicated for 4 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging. Band intensities are relative numbers representing intensities of the band areas.

Figure 30: IO-DNA complex formation in nuclear extracts

Reactions were carried out in 15 μ l volumes containing pre-annealed 180 nM IOs and 20 nM target DNA where indicated, nuclear extracts (NE) were added to the reactions as indicated. Reactions were incubated at 37°C for 10 min, and then an-

other 60 min upon addition of 1.125 μ l 10% SDS and 37.5 μ g Proteinase K. Binding was assayed by electrophoresis in a 7 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

5 **Figure 31: Nuclear factors favour IO-DNA complex formation by IO pairs**

Reactions were carried out in 15 μ l volumes containing 180 nM IOs and 20 nM target DNA. 10 μ g HeLa nuclear extract were added to the reactions. Reactions were incubated at 37°C for 10 min, and then another 60 min upon addition of 1.125 μ l 10% SDS and 37.5 μ g Proteinase K. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging

Figure 32: Chemical structures of LNA and INA **P nucleotide monomers.** B = nucleobase.

15 **Figure 33: Melting temperature data of INAs with different insertion patterns when hybridised to the complementary structure and LNA targets.** **P** = INA monomer **P**. T^L and $^{Me}C^L$ are locked nucleotides of thymine and 5-methylcytosine, respectively.

20 **Figure 34: Transition temperatures, T_m (°C) for hairpin probes with ssDNA targets.** T^L and $^{Me}C^L$ are locked nucleotides of thymine and 5-methylcytosine, respectively.

25 **Figure 35: A) transition curves of the non-intercalating pseudonucleotide comprising probes B) Two LNA probes comprising one intercalating pseudonucleotide together with the unmodified reference duplex. C) LNA probes comprising one or two intercalating pseudonucleotide together with the unmodified reference duplex. D) A non-intercalating pseudonucleotide comprising LNA probes and two probes comprising one intercalating pseudonucleotide together with corresponding DNA probe all hybridized to a target sequence comprising one intercalating pseudonucleotide..**

30 **Figure 36: Scheme 1. Schematic presentation of the conformations formed by T_4 -LNA oligonucleotides at transition temperature.**

Figure 37: Synthesis of 1'-aza pyrenmethyl pseudonucleotide

Figure 38: Sequences and hybridisation data of synthesized ODNs in DNA/DNA(RNA) duplexes

Figure 39: Hybridisation data for DNA Three-Way Junction

Figure 40: illustrates a beacon primer

Figure 41: - illustrates a PCR quantification strategy using beacon primers

Figure 42: illustrates complete complementarity and mismatch/excimer formation

Detailed description of the invention

Nucleic acids

The term "nucleic acid" covers the naturally occurring nucleic acids, DNA and RNA, including naturally occurring derivatives of DNA and RNA such as but not limited to methylated DNA, DNA containing adducts and RNA covalently bound to proteins. The term "nucleic acid analogues" covers synthetic derivatives and analogues of the naturally occurring nucleic acids, DNA and RNA. Synthetic analogues comprise one or more nucleotide analogues. The term "nucleotide analogue" comprises all nucleotide analogues capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing (see herein below), essentially like naturally occurring nucleotides.

Hence the terms "nucleic acids" or "nucleic acid analogues" designates any molecule, which essentially consists of a plurality of nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Intercalator pseudonucleotides are described in detail herein below. Nucleic acids or nucleic acid analogues according to the present invention may comprise more different nucleotides and nucleotide analogues with different backbone monomer units (see herein below).

Preferably, single strands of nucleic acids or nucleic acid analogues according to the present invention are capable of hybridising with a substantially complementary single stranded nucleic acid and/or nucleic acid analogue to form a double stranded nucleic acid or nucleic acid analogue. More preferably such a double stranded analogue is capable of forming a double helix. Preferably, the double helix is formed due to hydrogen bonding, more preferably, the double helix is a double helix selected from the group consisting of double helices of A form, B form, Z form and intermediates thereof.

Hence, nucleic acids and nucleic acid analogues according to the present invention includes, but is not limited to the kind of nucleic acids and/or nucleic acid analogues selected from DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA and mixtures thereof and hybrids thereof, as well as phosphorous atom modifications thereof, such as but not limited to phosphorothioates, methyl phospholates, phosphoramidates, phosphorodithiates, phosphorosele-
noates, phosphotriesters and phosphoboranoates. In addition non-phosphorous containing compounds may be used for linking to nucleotides such as but not limited to methyliminomethyl, formacetate, thioformacetate and linking groups comprising amides. In particular nucleic acids and nucleic acid analogues may comprise one or more intercalator pseudonucleotides according to the present invention.

Within this context "mixture" is meant to cover a nucleic acid or nucleic acid analogue strand comprising different kinds of nucleotides or nucleotide analogues. Furthermore, within this context, "hybrid" is meant to cover nucleic acids or nucleic acid analogues comprising one strand which comprises nucleotides or nucleotide analogues with one or more kinds of backbone and another strand which comprises nucleotides or nucleotide analogues with different kinds of backbone. By the term "duplex" is meant the hybridisation product of two strands of nucleic acids and/or nucleic acid analogues, wherein the strands preferably are of the same kind of nucleic acids and/or nucleic acid analogues.

By HNA is meant nucleic acids as for example described by Van Aetschot et al., 1995. By MNA is meant nucleic acids as described by Hossain et al, 1998. ANA refers to nucleic acids described by Allert et al, 1999. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. More preferably LNA is a nucleic acid as described in Singh et al, 1998, Koshkin et al, 1998 or Obika et al., 1997. PNA refers to peptide nucleic acids as for example described by Nielsen et al., 1991.

The term nucleotide designates the building blocks of nucleic acids or nucleic acid analogues and the term nucleotide covers naturally occurring nucleotides and derivatives thereof as well as nucleotides capable of performing essentially the same functions as naturally occurring nucleotides and derivatives thereof. Naturally occurring nucleotides comprise deoxyribonucleotides comprising one of the four nucleobases adenine (A), thymine (T), guanine (G) or cytosine (C), and ribonucleotides comprising one of the four nucleobases adenine (A), uracil (U), guanine (G) or cytosine (C).

Nucleotide analogues may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing.

Non-naturally occurring nucleotides according to the present invention includes, but is not limited to the nucleotides selected from PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, and α -D-RNA, β -D-RNA

The function of nucleotides and nucleotide analogues according to the present invention is to be able to interact specifically with complementary nucleotides via hydrogen bonding of the nucleobases of said complementary nucleotides as well as to be able to be incorporated into a nucleic acid or nucleic acid analogue. Naturally occurring nucleotides, as well as some nucleotide analogues are capable of being enzymatically incorporated into a nucleic acid or nucleic acid analogue, for example

by RNA or DNA polymerases, however nucleotides or nucleotide analogues may also be chemically incorporated into a nucleic acid or nucleic acid analogue.

5 Furthermore nucleic acids or nucleic acid analogues may be prepared by coupling two smaller nucleic acids or nucleic acid analogues to another, for example this may be done enzymatically by ligases or it may be done chemically.

10 Nucleotides or nucleotide analogues comprise a backbone monomer unit and a nucleobase. The nucleobase may be a naturally occurring nucleobase or a derivative thereof or an analogue thereof capable of performing essentially the same function. The function of a nucleobase is to be capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

15 Base pairing results in a specific hybridisation between predetermined and complementary nucleotides. Complementary nucleotides according to the present invention are nucleotides that comprise nucleobases that are capable of base-pairing.

20 Of the naturally occurring nucleobases adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, e.g. a nucleotide comprising A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

25 Nucleotides according to the present invention may further be derivatised to comprise an appended molecular entity. The nucleotides can be derivatised on the nucleobases or on the backbone monomer unit. Preferred sites of derivatisation on the bases include the 8-position of adenine, the 5-position of uracil, the 5- or 6-position of cytosine, and the 7-position of guanine. The heterocyclic modifications can be grouped into three structural classes: Enhanced base stacking, additional hydrogen bonding and the combination of these. Modifications that enhance base stacking by expanding the π -electron cloud of planar systems are represented by

30 conjugated, lipophilic modifications in the 5-position of pyrimidines and the 7-

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position of 7-deaza-purines. Substitutions in the 5-position of pyrimidines modifications include propynes, hexynes, thiazoles and simply a methyl group; and substituents in the 7-position of 7-deaza purines include iodo, propynyl, and cyano groups. It is also possible to modify the 5-position of cytosine from propynes to five-membered heterocycles and to tricyclic fused systems, which emanate from the 4- and 5-position (cytosine clamps). A second type of heterocycle modification is represented by the 2-amino-adenine where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified adenine and the tricyclic cytosine analog having an ethoxyamino functional group of heteroduplexes. Furthermore, N2-modified 2-amino adenine modified oligonucleotides are among commonly modifications. Preferred sites of derivatisation on ribose or deoxyribose moieties are modifications of nonconnecting carbon positions C-2' and C-4', modifications of connecting carbons C-1', C-3' and C-5', replacement of sugar oxygen, O-4', Anhydro sugar modifications (conformational restricted), cyclosugar modifications (conformational restricted), ribofuranosyl ring size change, connection sites – sugar to sugar, (C-3' to C-5'/ C-2' to C-5'), hetero-atom ring – modified sugars and combinations of above modifications.. However, other sites may be derivatised, as long as the overall base pairing specificity of a nucleic acid or nucleic acid analogue is not disrupted. Finally, when the backbone monomer unit comprises a phosphate group, the phosphates of some backbone monomer units may be derivatised.

Oligonucleotide or oligonucleotide analogue as used herein are molecules essentially consisting of a sequence of nucleotides and/or nucleotide analogues and/or intercalator pseudo-nucleotides. Preferably oligonucleotide or oligonucleotide analogue comprises 3-200, 5-100, 10-50 individual nucleotides and/or nucleotide analogues and/or intercalator pseudo-nucleotides, as defined above.

Target nucleic acids

A target nucleic acid or target nucleic acid analogue sequence refers to a nucleotide or nucleotide analogue sequence which comprise one or more sites/sequences for hybridisation of one or more oligonucleotide(s) and/or oligonucleotide analogue(s), for example primers or probes. Target sequences may be found in any nucleic acid

or nucleic acid analogue including, but not limited too, other probes, RNA, genomic DNA, plasmid DNA, cDNA and can for example comprise a wild-type or mutant gene sequence or a regulatory sequence thereof or an amplified nucleic acid sequence, for example as when amplified by PCR. A target sequence may be of any length. The site addressed may or may not be one contiguous sequence. For example said site may be composed of two or more contiguous subsequences separated by any number of nucleotides and/or nucleotide analogues. Preferentially the total length of the site addressed, composed by all subsequences on that particular target nucleic acid or target nucleic acid analogue, by said oligonucleotide and/or oligonucleotide analogue, typically is less than 100 nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides.

Homologous nucleic acids

Nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are said to be homologously complementary, when they are capable of hybridising. Preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under low stringency conditions, more preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under medium stringency conditions, more preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under high stringency conditions.

High stringency conditions as used herein shall denote stringency as in comparison to, or at least as stringent as, what is normally applied in connection with Southern blotting and hybridisation as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al.,

1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

5 Medium stringency conditions as used herein shall denote hybridisation in a buffer containing 1 mM EDTA, 10mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 140 mM NaCl, at pH 7.0, or a buffer similar to this having approximately the same impact on hybridization stringency. Preferably, around 1,5 μM of each nucleic acid or nucleic acid analogue strand is provided. Alternatively medium stringency may denote hybridisation in a buffer containing 50 mM KCl, 10 mM TRIS-HCl (pH 9,0), 0.1% Triton X-100, 2 mM MgCl_2 .

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Low stringency conditions according to the present invention denote hybridisation in a buffer constituting 1 M NaCl, 10 mM Na_3PO_4 at pH 7,0, or a buffer similar to this having approximately the same impact on hybridization stringency.

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Alternatively, homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues substantially complementary to each other over a given sequence, such as more than 70% complementary, for example more than 75% complementary, such as more than 80% complementary, 20 for example more than 85% complementary, such as more than 90% complementary, for example more than 92% complementary, such as more than 94% complementary, for example more than 95% complementary, such as more than 96% complementary, for example more than 97% complementary.

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Preferably said given sequence is at least 4 nucleotides long, for example at least 10 nucleotides, such as at least 15 nucleotides, for example at least 20 nucleotides, such as at least 25 nucleotides, for example at least 30 nucleotides, such as between 10 and 500 nucleotides, for example between 4 and 100 nucleotides long, such as between 10 and 50 nucleotides long. More preferably homologously complementary oligonucleotides or oligonucleotide analogues are substantially homologously complementary over their entire length.

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Specificity of hybridisation

The specificity of hybridisation of nucleic acids and/or nucleic acid analogues and/or oligonucleotides and/or oligonucleotide analogues refers to the ability of which said hybridisation event distinguishes between homologously complementary hybridisation partners according to their sequence differences under given stringency conditions. Often it is the intention to target only one particular sequence (the target sequence) in a mixture of nucleic acids and/or nucleic acid analogues and/or oligonucleotides and/or oligonucleotide analogues and to avoid hybridization to other sequences even though they have strong similarity to said target sequence. Sometimes only one or few nucleotides differ among target and non-target sequences in the sequence-region used for hybridization.

High specificity in hybridisation as used herein denotes hybridisation under high stringency conditions at which an oligonucleotide or oligonucleotide analogue will hybridise with a homologous target sequence significantly better than to a nearly identical sequence differing only from said target sequence by one or few base-substitutions.

Discrimination

Discrimination refers to the ability of oligonucleotides and/or oligonucleotide analogues, in a sequence-independent manner, to hybridise preferentially with either RNA or DNA. Accordingly, the melting temperature of a hybrid consisting of oligonucleotide and/or oligonucleotide analogue and a homologously complementary RNA (RNA hybrid) is either significantly higher or lower than the melting temperature of a hybrid between said oligonucleotide and/or oligonucleotide analogue and a homologously complementary DNA (DNA hybrid).

RNA-like and DNA-like

RNA-like refers to nucleic acid analogues or oligonucleotide analogues behaving like RNA with respect to hybridisation to homologously complementary oligonucleotides and/or oligonucleotide analogues comprising at least one internal pseudonucleotide. Accordingly, RNA-like nucleic acid analogues or oligonucleotide analogues can be functionally categorized on the basis of their ability to hybridise with oligonucleotides and/or oligonucleotide analogues able to discriminate between RNA and

DNA. Preferentially, said oligonucleotide analogues able to discriminate between RNA and DNA comprises one or more internally positioned pseudonucleotide intercalators and consequently, said oligonucleotide analogue comprising pseudonucleotide intercalators will preferentially not hybridise to said RNA-like nucleic acid analogues or oligonucleotide analogues.

Examples of RNA-like molecules are RNA, 2'-O-methyl RNA, LNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, 2'-R-RNA, 2'-OR-RNA, and mixtures thereof.

Likewise, DNA-like refers to nucleic acid analogues or oligonucleotide analogues behaving like DNA with respect to hybridisation to homologously complementary nucleic acids and/or nucleic acid analogues. Accordingly, DNA-like nucleic acids or nucleic acid analogues can be functionally categorized on the basis of their ability to hybridise with oligonucleotides or oligonucleotide analogues able to discriminate between RNA and DNA. Preferentially, said oligonucleotides or oligonucleotide analogues able to discriminate between RNA and DNA comprises one or more internally positioned pseudonucleotide intercalators; and consequently, said oligonucleotide analogue comprising pseudonucleotide intercalators will preferentially hybridise to said DNA-like nucleic acid analogues or oligonucleotide analogues.

Examples of DNA-like molecules is DNA and INA (Christensen, 2002. Intercalating nucleic acids containing insertions of 1-O-(1-pyrenylmethyl)glycerol: stabilisation of dsDNA and discrimination of DNA over RNA. Nucl. Acids. Res. 2002 30: 4918-4925).

Cross-hybridisation

The term cross-hybridisation covers unattended hybridisation between at least two nucleic acids and/or nucleic acid analogues, i.e. cross-hybridisation may also be denoted intermolecular hybridisation. Hence the term cross-hybridization may be used to describe the hybridisation of for example a nucleic acid probe or nucleic acid analogue probe sequence to other nucleic acid sequences and/or nucleic acid analogue sequences than its intended target sequence.

Often cross-hybridization occurs between a probe and one or more homologously complementary non-target sequences, even though these have a lower degree of complementarity than the probe and its complementary target sequence. This unwanted effect could be due to a large excess of probe over target and/or fast annealing kinetics. Cross-hybridization also occurs by hydrogen bonding between few nucleobase pairs, e.g. between primers in a PCR reaction, resulting in primer dimer formation and/ or formation of unspecific PCR products.

Especially nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to form dimer or higher order complexes based on base pairing. Especially probes comprising nucleotide analogues such as, but not limited to, DNA, RNA, 2'-O-methyl RNA, PNA, HNA, MNA, ANA, LNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, 2'-R-RNA, 2'-OR-RNA, and mixtures thereof generally have a high affinity for hybridising to other oligonucleotide analogues comprising backbone monomer units of the same type. Hence even though individual probe molecules only have a low degree of complementarity, they tend to hybridise.

Self-hybridisation

The term self-hybridisation covers the process wherein a nucleic acid or nucleic acid analogue molecule anneals to itself by folding back on itself, generating a secondary structure like for example a hairpin structure, i.e. self-hybridisation may also be defined as intramolecular hybridisation. In most applications it is of importance to avoid self-hybridization. The generation of said secondary structures may inhibit hybridization with desired nucleic acid target sequences. This is undesired in most assays for example when the nucleic acid or nucleic acid analogue is used as primer in PCR reactions or as fluorophore/ quencher labeled probe for exonuclease assays. In both assays self-hybridisation will inhibit hybridization to the target nucleic acid and additionally the degree of fluorophore quenching in the exonuclease assay is lowered.

Especially nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to self-hybridise. Especially probes comprising nucleotide analogues such as, but not limited to, DNA, RNA, 2'-O-methyl RNA, PNA, HNA, MNA, ANA, LNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, 2'-R-RNA, 2'-OR-RNA generally have a high affinity for self-hybridising. Hence even though individual probe molecules only have a low degree of self-complementarity they tend to self-hybridise.

Melting temperature

Melting of nucleic acids refer to thermal separation of the two strands of a double-stranded nucleic acid molecule.

The melting temperature (T_m) denotes the temperature in degrees centigrade at which 50% helical (hybridised) versus coil (unhybridised) forms are present.

A high melting temperature is indicative of a stable complex and accordingly of a high affinity between the individual strands. Vice versa a low melting temperature is indicative of a relatively low affinity between the individual strands. Accordingly, usually strong hydrogen bonding between the two strands results in a high melting temperature.

Furthermore, as disclosed by the present invention, intercalation of an intercalator between nucleobases of a double stranded nucleic acid may also stabilise double stranded nucleic acids and accordingly result in a higher melting temperature.

In addition the melting temperature is dependent on the physical/chemical state of the surroundings. For example the melting temperature is dependent on salt concentration and pH.

The melting temperature may be determined by a number of assays, for example it may be determined by using the UV spectrum to determine the formation and breakdown (melting) of hybridisation.

Backbone monomer unit

The backbone monomer unit of a nucleotide or a nucleotide analogue or an intercalator pseudonucleotide according to the present invention is the part of the nucleotide, which is involved in incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide into the backbone of a nucleic acid or a nucleic acid analogue. Any suitable backbone monomer unit may be employed with the present invention.

In particular the backbone monomer unit of intercalator pseudonucleotides according to the present invention may be selected from the backbone monomer units mentioned herein below.

The backbone monomer unit comprises the part of a nucleotide or nucleotide analogue or intercalator pseudonucleotide that may be incorporated into the backbone of an oligonucleotide or an oligonucleotide analogue. In addition, the backbone monomer unit may comprise one or more leaving groups, protecting groups and/or reactive groups, which may be removed or changed in any way during synthesis or subsequent to synthesis of an oligonucleotide or oligonucleotide analogue comprising said backbone monomer unit.

It is important to note that the term backbone monomer unit according to the present invention only includes the backbone monomer unit per se and it does not include for example a linker connecting a backbone monomer unit to an intercalator. Hence, the intercalator as well as the linker is not part of the backbone monomer unit.

Accordingly, backbone monomer units only include atoms, wherein the monomer is incorporated into a sequence, are selected from the group consisting of

- a) atoms which are capable of forming a linkage to the backbone monomer unit of a neighboring nucleotide; or
- b) atoms which at least at two sites are connected to other atoms of the backbone monomer unit; or
- c) atoms which at one site is connected to the backbone monomer unit and otherwise is not connected with other atoms

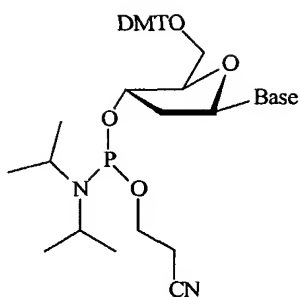
More preferably, backbone monomer unit atoms are thus defined as the atoms involved in the direct linkage (shortest path) between the backbone Phosphor-atoms of neighbouring nucleotides, when the monomer is incorporated into a sequence, wherein the neighbouring nucleotides are naturally occurring nucleotides,.

The backbone monomer unit may be any suitable backbone monomer unit. In one embodiment of the present invention, the backbone monomer unit may for example be selected from the group consisting of the backbone monomer units of DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, α -L-RNA or α -D-RNA, β -D-RNA.

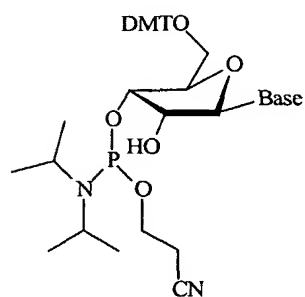
Below is depicted a range of different backbone monomer units of nucleotides and nucleotide analogues, and how they are connected to the nucleobases via linkers that are attached at one or two positions of the backbone monomer unit:

Examples of oligomers of DNA, RNA & PNA

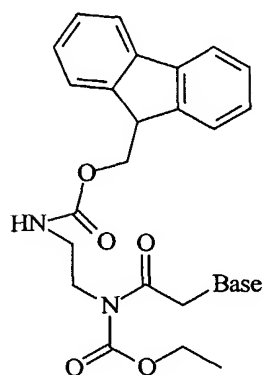
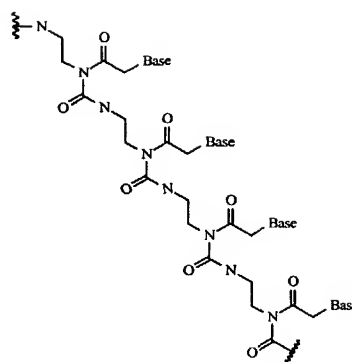
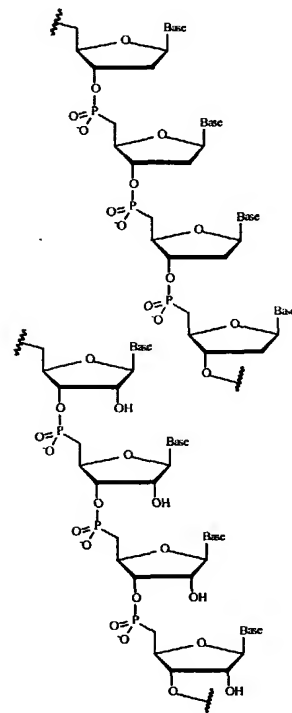
DNA



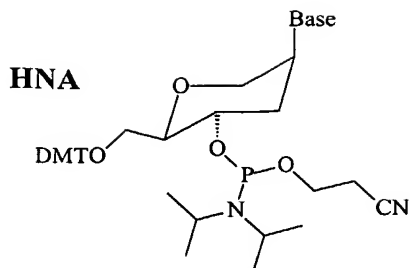
RNA



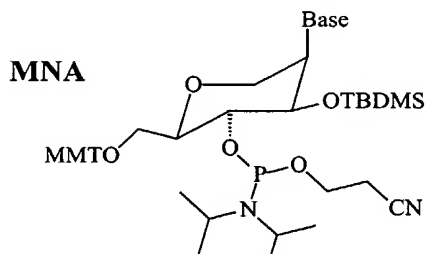
PNA

Ref. Nielsen, P. E. et al. *Science*, 1991, 254, 1497.

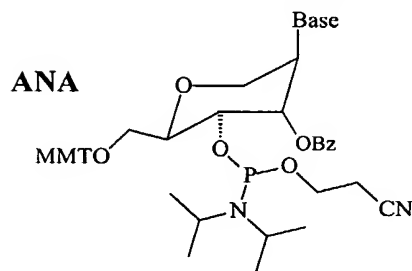
Examples of oligomers of some analogues



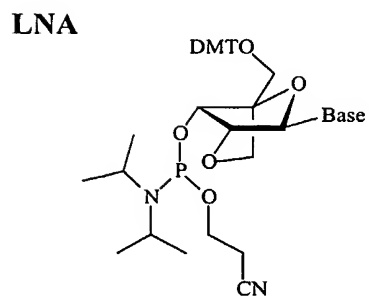
Ref. Van Aerschot, A. et al. *Angew. Chem. Int. Ed., Engl.*, 1995, 34, 1338-1339.



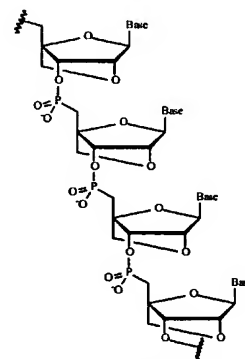
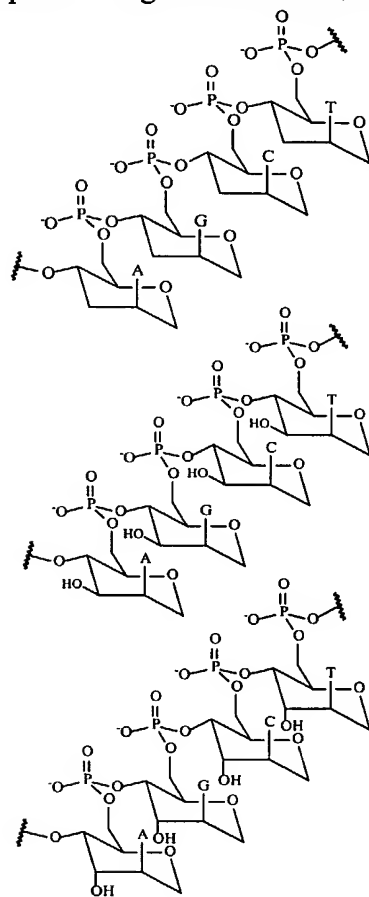
Ref. Hossain N. et al. *J. Org. Chem.*, 1998, 63, 1574-1582.



Ref. Allart, B. et al. *Chem. Eur. J.*, 1999, 5, 2424-2431.

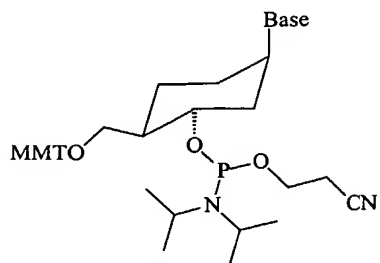


Ref. Singh, S. K. et al. *Chem. Commun.*, 1998, 455-456; Koshkin, A. A. et al. *Tetrahedron*, 1998, 54, 3607-3630; Obika, S. et al. *Tetrahedron Lett.*, 1997, 38, 8735-8738.



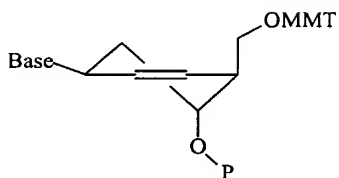
Examples of oligomers of some analogues

Cyclohexanyl-NA (CNA)

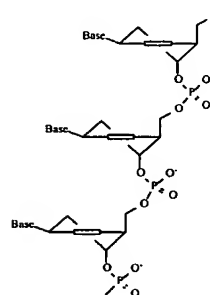
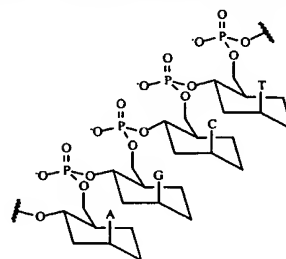


Ref: Maurinsh, Y.; et al. Chem. Eur. J., 1999, 2139-2150.

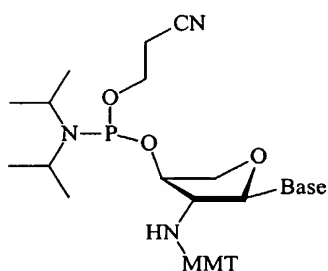
Cyclohexenyl-NA (CeNA)



Ref: Wang, J.; et al. J. Am. Chem. Soc., 2000, 8595-8602.

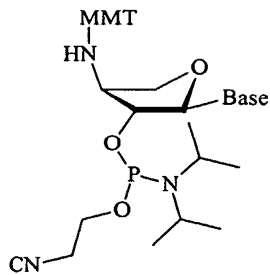


(2'-NH)-TNA

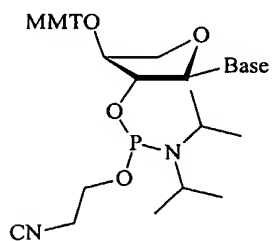


Ref: Wu, X. et al., Org. Lett., 2002, 4, 1279-1282

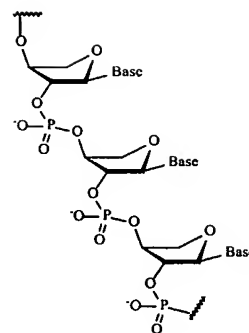
(3'-NH)-TNA



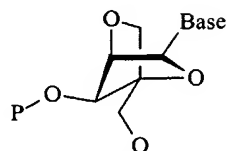
TNA



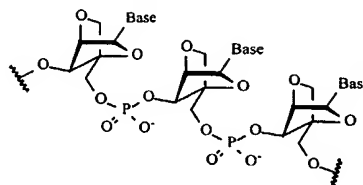
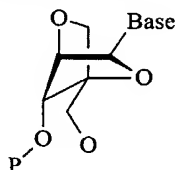
Ref: Wu, X. et al., Org. Lett., 2002, 4, 1279-1282



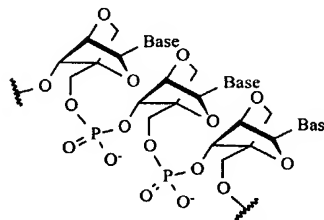
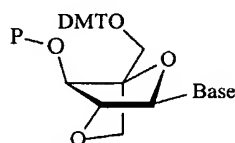
Section of a nucleic acid of the respective analogues

 α -L-Ribo-LNA

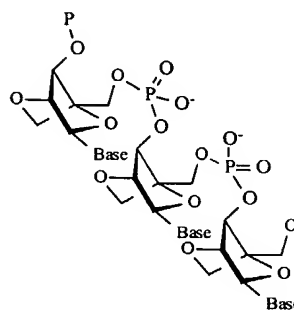
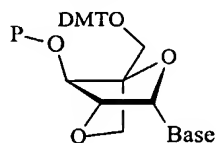
Ref: Rajwanshi, V. K. et al. Chem. Commun., 1999, 1395-1396.

 α -L-Xylo-LNA

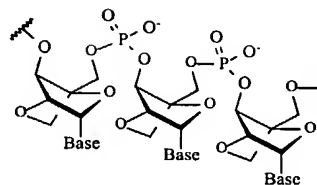
Ref: Rajwanshi, V. K et al. Angew. Chem. Int. Ed., 2000, 1656-1659.

 β -D-Xylo-LNA

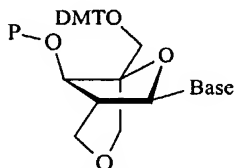
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 α -D-Ribo-LNA

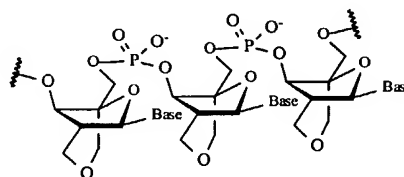
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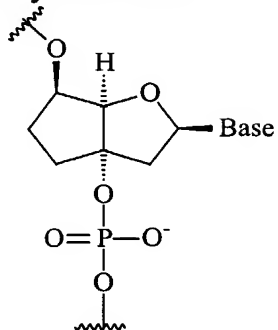
[3.2.1]-LNA



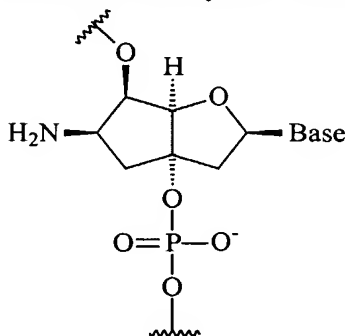
Ref: Wang, G.; et al. Tetrahedron, 1999, 7707-2724.



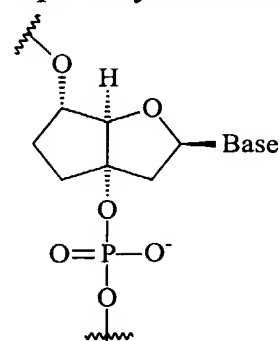
Bicyclo-DNA



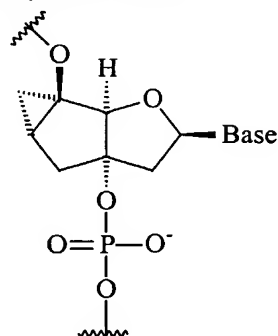
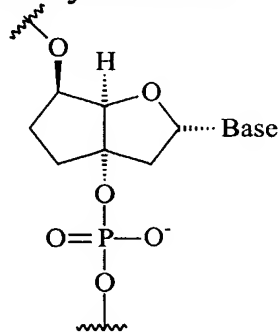
6'-Amino-Bicyclo-DNA



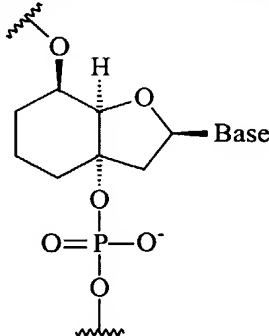
5'-epi-Bicyclo-DNA



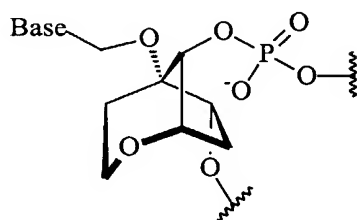
Tricyclo-DNA

 α -Bicyclo-DNA

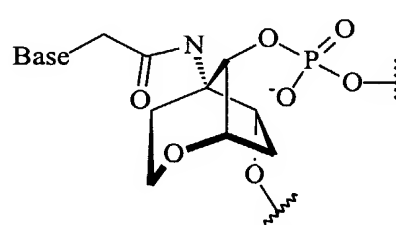
Bicyclo[4.3.0]-DNA



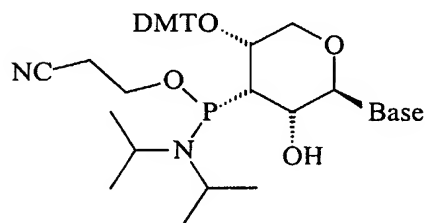
Bicyclo[3.2.1]-DNA



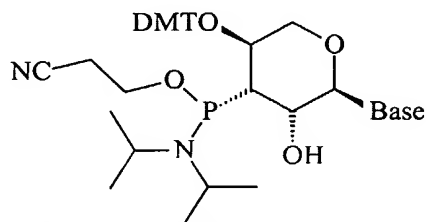
Bicyclo[3.2.1]amide-DNA



Ref: All of the Bicyclo-DNAs are reviewed in Leumann, C. J., Bioorg. Med. Chem., 2002, 841-854.

 β -D-Ribopyranosyl-NA

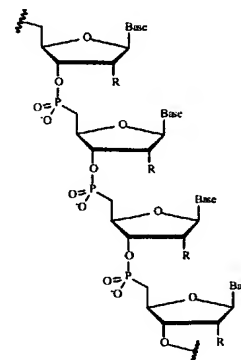
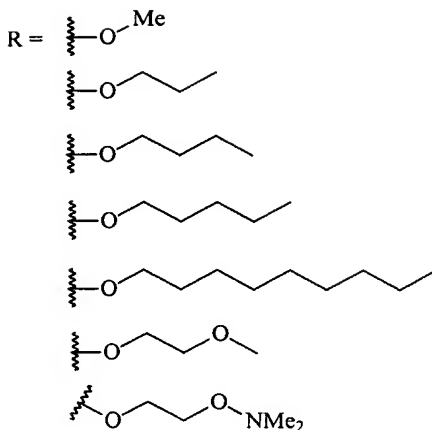
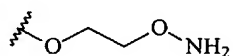
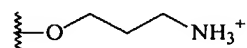
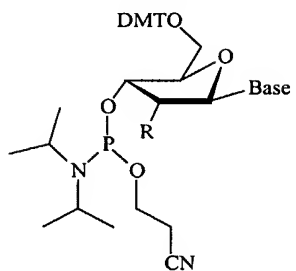
Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

 α -L-Lyxopyranosyl-NA

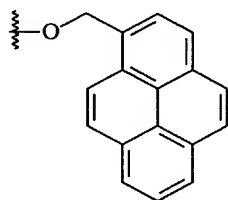
Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

2'-R-RNA

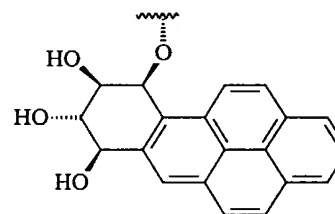
General structure of 2'-modified oligomers



Ref: Reviewed by Manoharan, M. Biochim. BioPhys. Acta, 1999, 117-130.

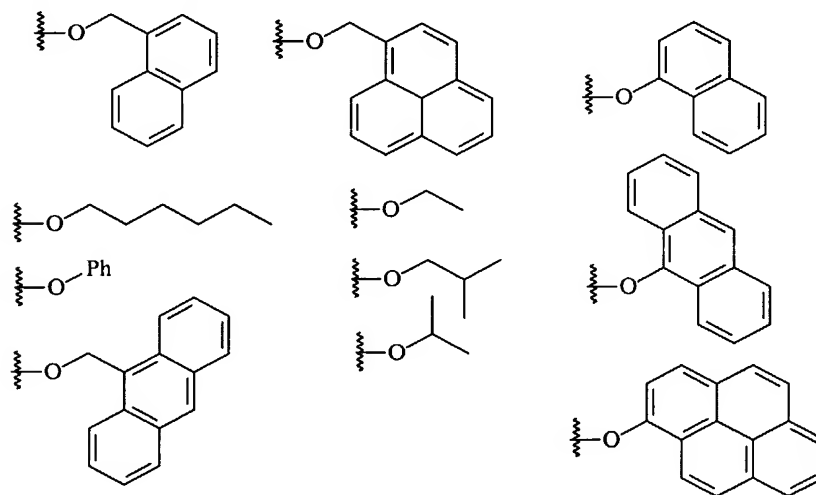


Ref: Yamana, K. et al., Tetrahedron Lett., 1991, 6347-6350.



Ref: Sayer, J. et al., J. Org. Chem., 1991, 56, 20-29.

Examples of modifications that, to our knowlegde, are not synthesised or published yet:

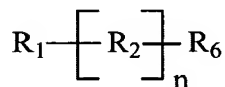


5

The backbone monomer unit of LNA (locked nucleic acid) is a sterically restricted DNA backbone monomer unit, which comprises an intramolecular bridge that restricts the usual conformational freedom of a DNA backbone monomer unit. LNA may be any LNA molecule as described in WO 99/14226 (Exigon), preferably, LNA

The backbone monomer unit of LNA (locked nucleic acid) is a sterically restricted DNA backbone monomer unit, which comprises an intramolecular bridge that restricts the usual conformational freedom of a DNA backbone monomer unit. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. Preferred LNA according to the present invention comprises a methyl linker connecting the 2'-O position to the 4'-C position, however other LNA's such as LNA's wherein the 2' oxy atom is replaced by either nitrogen or sulphur are also comprised within the present invention.

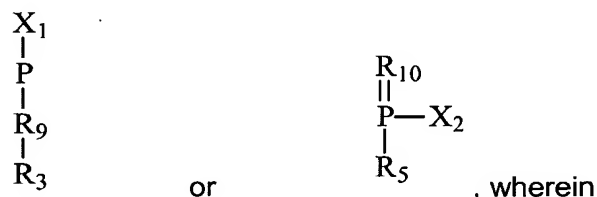
The backbone monomer unit of intercalator pseudonucleotides according to present invention preferably have the general structure before being incorporated into an oligonucleotide and/or nucleotide analogue:



wherein

$n = 1$ to 6, preferably $n = 2$ to 6, more preferably $n = 3$ to 6, more preferably $n = 2$ to 5, more preferably $n = 3$ to 5, more preferably $n = 3$ to 4.

R_1 is a trivalent or pentavalent substituted phosphoratom, preferably R_1 is



R_2 may individually be selected from an atom capable of forming at least two bonds, said atom optionally being individually substituted, preferably R_2 is individually selected from O, S, N, C, P, optionally individually substituted. By the term "individually" is meant that R_2 can represent one, two or more different groups in the same

molecule. The bonds between two R_2 may be saturated or unsaturated or a part of a ring system or a combination thereof

Each R_2 may individually be substituted with any suitable substituent, such as a
5 substituent selected from H, lower alkyl, C2-6 alkenyl, C6-10 aryl, C7-11 arylmethyl, C2-7 acyloxymethyl, C3-8 alkoxycarbonyloxymethyl, C7-11 aryloxymethyl, C3-8 S-acyl-2-thioethyl;

An "alkyl" group refers to an optionally substituted saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the
10 alkyl group has 1 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkyl of from 1 to 12 carbons, more preferably 1 to 6 carbons, more preferably 1 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

15 An "alkenyl" group refers to an optionally substituted hydrocarbon containing at least one double bond, including straight-chain, branched-chain, and cyclic alkenyl groups, all of which may be optionally substituted. Preferably, the alkenyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkenyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms
20 are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

An "alkynyl" group refers to an optionally substituted unsaturated hydrocarbon containing at least one triple bond, including straight-chain, branched-chain, and cyclic
25 alkynyl groups, all of which may be optionally substituted. Preferably, the alkynyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkynyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

30 An "aryl" refers to an optionally substituted aromatic group having at least one ring with a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl, biaryl, and triaryl groups. Examples of aryl substitution substituents include alkyl,

alkenyl, alkynyl, aryl, amino, substituted amino, carboxy, hydroxy, alkoxy, nitro, sulfonyl, halogen, thiol and aryloxy.

5 A "carbocyclic aryl" refers to an aryl where all the atoms on the aromatic ring are carbon atoms. The carbon atoms are optionally substituted as described above for an aryl. Preferably, the carbocyclic aryl is an optionally substituted phenyl.

10 A "heterocyclic aryl" refers to an aryl having 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen. Examples of heterocyclic aryls include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, and imidazolyl. The heterocyclic aryl is optionally substituted as described above for an aryl.

The substituents on two or more R_2 may alternatively join to form a ring system, such as any of the ring systems as defined above.

15 Preferably R_2 is substituted with an atom or a group selected from H, methyl, R_4 , hydroxyl, halogen, and amino, more preferably R_2 is substituted with an atom or a group selected from H, methyl, R_4 .

More preferably R_2 is individually selected from O, S, NH, N(Me), N(R_4), C(R_4)₂, CH(R_4) or CH₂, wherein R_4 is as defined below,

20

R_3 = methyl, beta-cyanoethyl, p-nitrophenetyl, o-chlorophenyl, or p-chlorophenyl.

25 R_4 = lower alkyl, preferably lower alkyl such as methyl, ethyl, or isopropyl, or heterocyclic, such as morpholino, pyrrolidino, or 2,2,6,6-tetramethylpyrrolidino, wherein lower alkyl is defined as C₁ - C₆, such as C₁ - C₄.

R_5 = alkyl, alkoxy, aryl or H, with the proviso that R_5 is H when $X_2 = O^-$, preferably R_5 is selected from lower alkyl, lower alkoxy, aryloxy. In a preferred embodiment aryloxy is selected from phenyl, naphthyl or pyridine.

30 R_6 is a protecting group, selected from any suitable protecting groups. Preferably R_6 is selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups

mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl (DMT).

5

R_9 is selected from O, S, N optionally substituted, preferably R_9 is selected from O, S, NH, N(Me).

R_{10} is selected from O, S, N, C, optionally substituted.

10

$X_1 = \text{Cl, Br, I, or } N(R_4)_2$

$X_2 = \text{Cl, Br, I, } N(R_4)_2, \text{ or } O^-$

15

As described above with respect to the substituents the backbone monomer unit can be acyclic or part of a ring system.

20

In one preferred embodiment of the present invention the backbone monomer unit of an intercalator pseudonucleotide is selected from the group consisting of acyclic backbone monomer units. Acyclic is meant to cover any backbone monomer unit, which does not comprise a ringstructure, for example the backbone monomer unit preferably does not comprise a ribose or a deoxyribose group.

25

In particular, it is preferred that the backbone monomer unit of an intercalator pseudonucleotide is an acyclic backbone monomer unit, which is capable of stabilising a bulge insertion (see herein below).

30

In another preferred embodiment the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group consisting of trivalent and pentavalent phosphorous atom such as a pentavalent phosphorous atom. More preferably the phosphate atom of the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group

35

consisting of, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

5 In particular it is preferred that the backbone monomer unit of an intercalator pseudonucleotide according to the present invention is selected from the group consisting of acyclic backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

10 Preferred backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups are backbone monomer units, wherein the distance from at least one phosphor atom to at least one phosphor atom of a neighbouring nucleotide, not including the phosphor atoms, is at the most 6
15 atoms long, for example 2, such as 3, for example 4, such as 5, for example 6 atoms long, when the backbone monomer unit is incorporated into a nucleic acid backbone.

20 The distance is measured as the direct linkage (i.e. the shortest path) as discussed above.

Preferably the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 5 atoms are separating the phosphor atom of the intercalator
25 pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 5 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, in both cases not including the phosphor atoms themselves.

30 Preferably the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 4 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 4 atoms are separating the phosphor atom of the intercalator

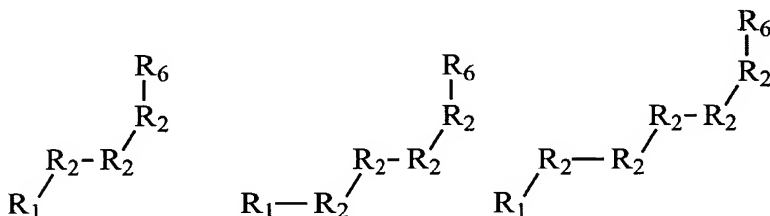
pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, in both cases not including the phosphor atoms themselves.

5 In a particularly preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit that comprises a phosphoramidit and more preferably the backbone monomer unit comprises a trivalent phosphoramidit.

10 Suitable trivalent phosphoramidits are trivalent phosphoramidits that may be incorporated into the backbone of a nucleic acid and/or a nucleic acid analogue. Usually, the amidit group per se may not be incorporated into the backbone of a nucleic acid, but rather the amidit group or part of the amidit group may serve as a leaving group and/ or protecting group. However, it is preferred that the backbone monomer unit comprises a phosphoramidit group, because such a group may
15 facilitate the incorporation of the backbone monomer unit into a nucleic acid backbone.

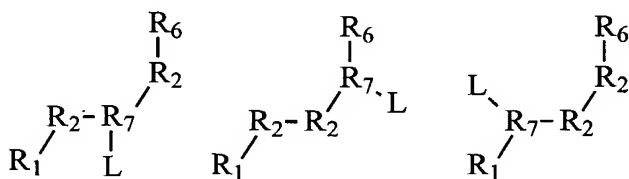
Preferably the acyclic backbone monomers may be selected from one of the general structures depicted below:

20

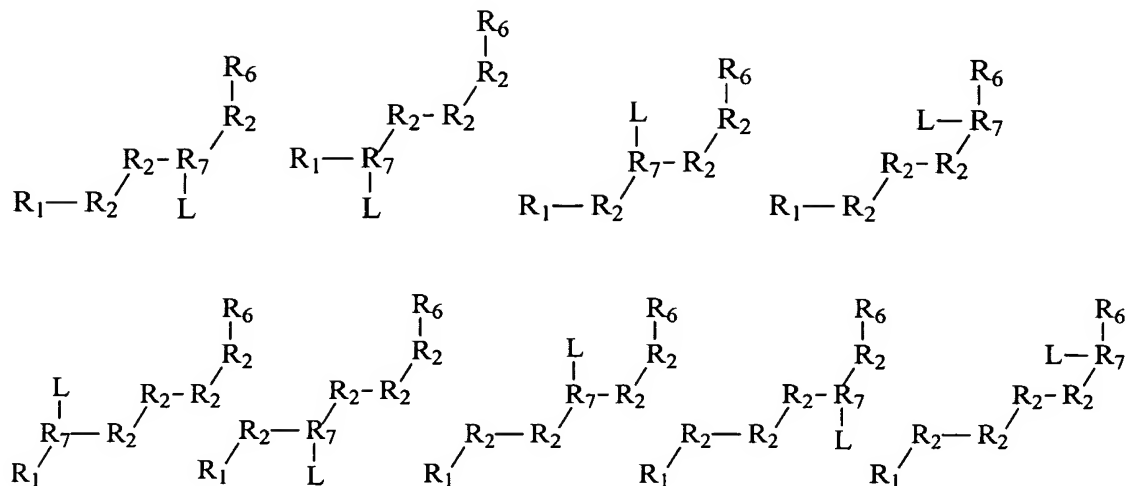


wherein R_1 , R_2 and R_6 are as defined above.

25 More preferably, the acyclic backbone monomer unit may be selected from the group depicted below:

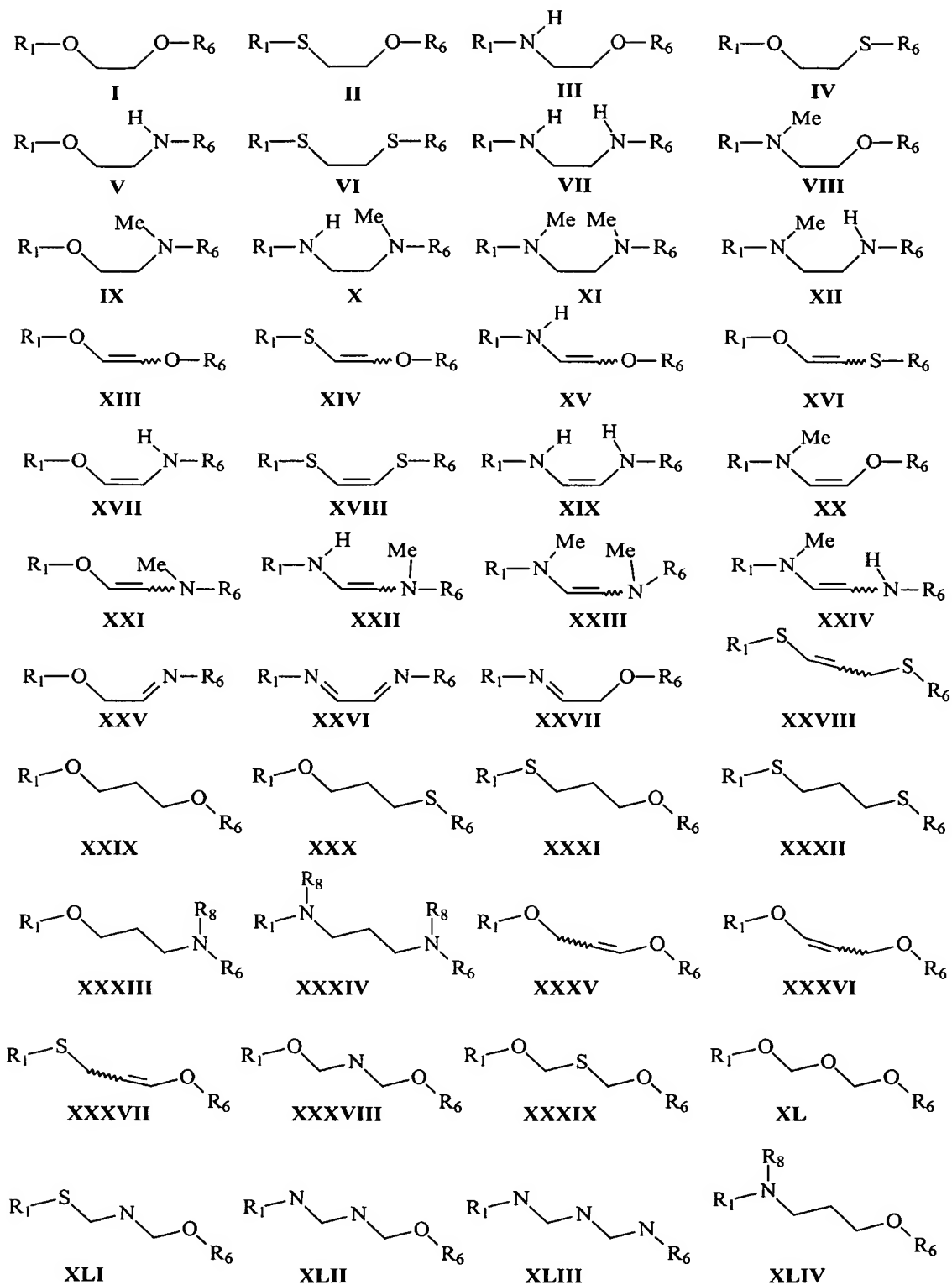


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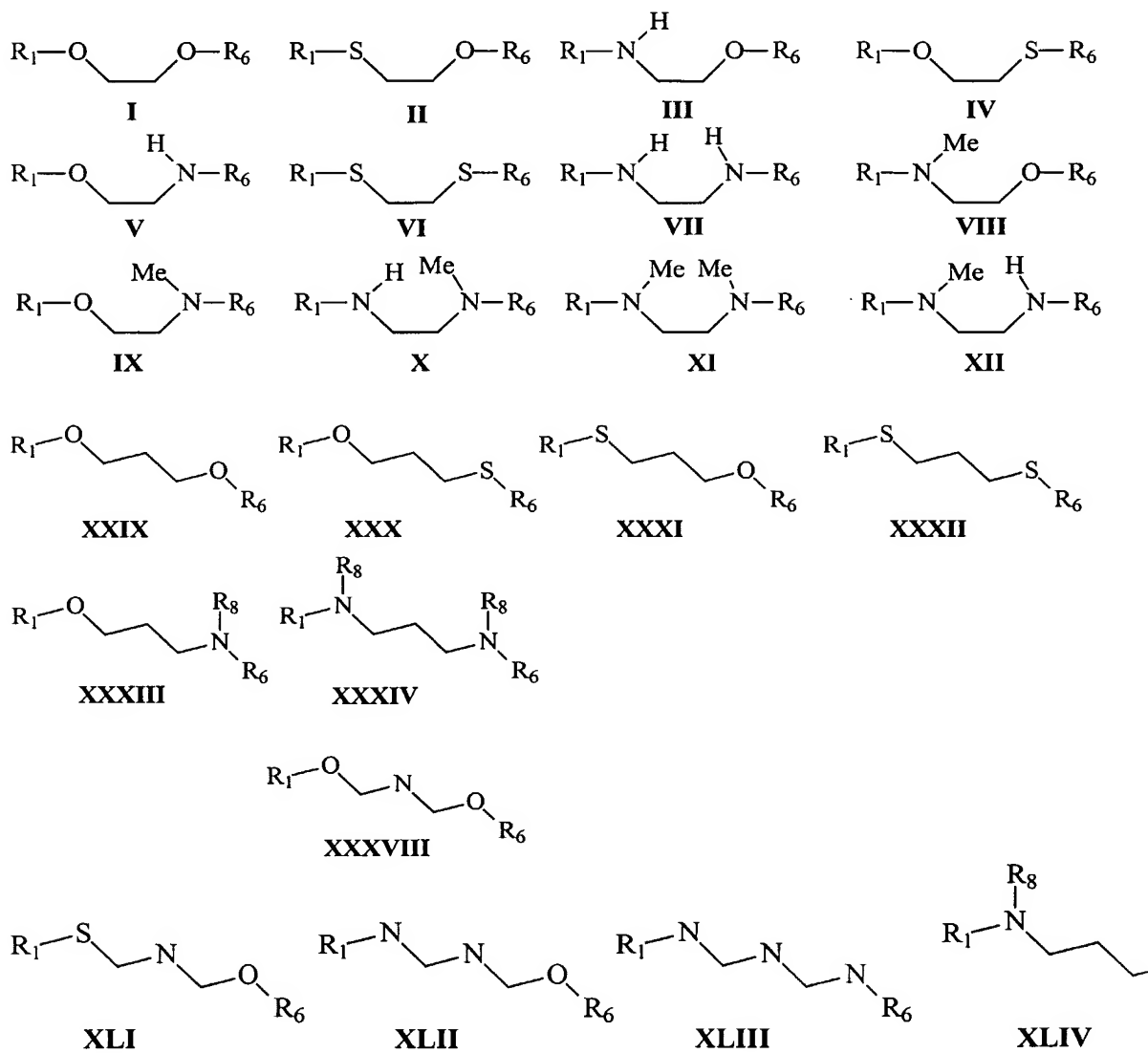
- 5 wherein R_1 , R_2 and R_6 are as defined above, and $R_7 = N$, or CH , and L is the linker.

Below are specific examples of backbone monomer units numbered I) to XLIV, wherein R_1 and R_6 are as defined above, and R_8 may be R_4 or H , optionally substituted.

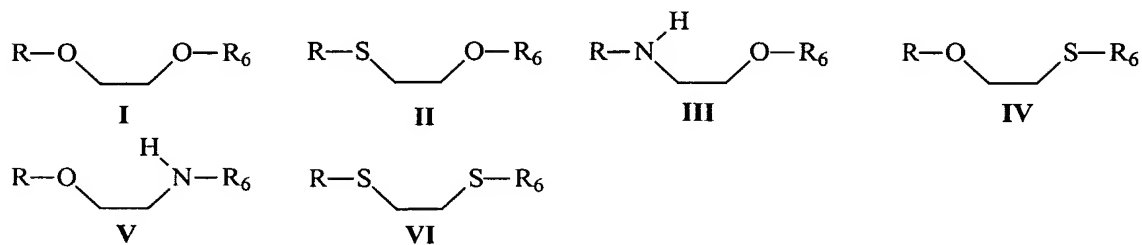


Me denotes methyl

Even more preferable the backbone monomer unit including optional protecting groups may be selected from the group consisting of the structures I) to XLIV) as indicated herein below:

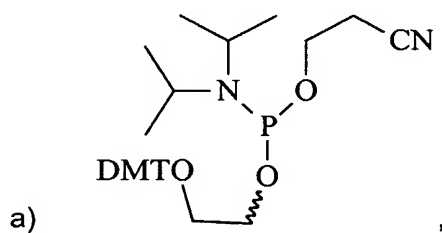


Most preferred are the backbone monomer units selected from the group consisting of:

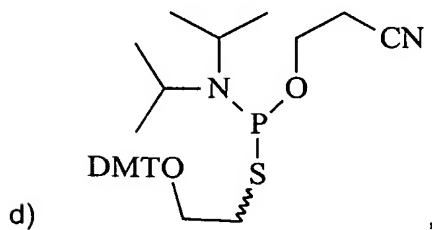
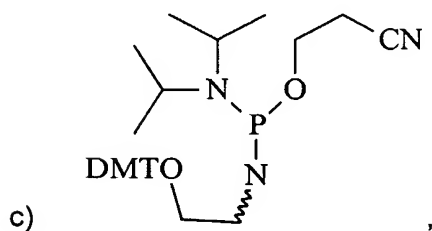
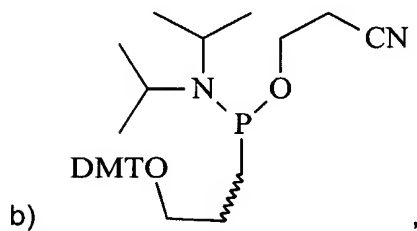


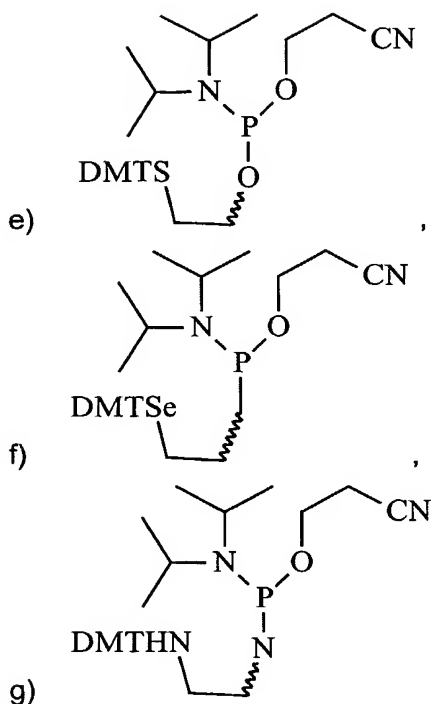
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Preferably, the acyclic backbone monomer unit may be selected from the group consisting of the structures a) to g) as indicated below:



10





5

The backbone monomer unit of an intercalator pseudonucleotide which is inserted into an oligonucleotide or oligonucleotide analogue, according to the present invention may comprise a phosphodiester bond. Additionally, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may comprise a pentavalent phosphoramidate. Preferably, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention is an acyclic backbone monomer unit that may comprise a pentavalent phosphoramidate.

10

Leaving group

15

The backbone monomer unit according to the present invention may comprise one or more leaving groups. Leaving groups are chemical groups, which are part of the backbone monomer unit when the intercalator pseudonucleotide or the nucleotide is a monomer, but which are no longer present in the molecule once the intercalator pseudonucleotide or the nucleotide has been incorporated into an oligonucleotide or oligonucleotide analogue.

20

The nature of a leaving group depends of the backbone monomer unit. For example, when the backbone monomer unit is a phosphor amidit, the leaving group, may for example be an diisopropylamine group. In general, when the backbone monomer unit is a phosphor amidit, a leaving group is attached to the phosphor atom for example in the form of diisopropylamine and said leaving group is removed upon coupling of the phosphor atom to a nucleophilic group, whereas the rest of the phosphate group, may become part of the nucleic acid or nucleic acid analogue backbone.

Reactive group

The backbone monomer units according to the present invention may furthermore comprise a reactive group which is capable of performing a chemical reaction with another nucleotide or oligonucleotide or nucleic acid or nucleic acid analogue to form a nucleic acid or nucleic acid analogue, which is one nucleotide longer than before the reaction.

Accordingly, when nucleotides are in their free form, i.e. not incorporated into a nucleic acid, they may comprise a reactive group capable of reacting with another nucleotide or a nucleic acid or nucleic acid analogue.

In preferred embodiments of the present invention said reactive group may be protected by a protecting group. Prior to said chemical reaction, said protection group may be removed. The protection group will thus not be a part of the newly formed nucleic acid or nucleic acid analogue.

Examples of reactive groups are nucleophiles such as the 5'-hydroxy group of DNA or RNA backbone monomer units.

Protecting group

The backbone monomer unit according to the present invention may also comprise a protecting group, which can be removed, and wherein removal of the protecting group allows for a chemical reaction between the intercalator pseudonucleotide and a nucleotide or nucleotide analogue or another intercalator pseudonucleotide.

In particular, a nucleotide monomer or nucleotide analogue monomer or intercalator pseudonucleotide monomer may comprise a protecting group, which is no longer present in the molecule once the nucleotide or nucleotide analogue or intercalator pseudonucleotide has been incorporated into a nucleic acid or nucleic acid analogue.

Furthermore, backbone monomer units may comprise protecting groups which may be present in the oligonucleotide or oligonucleotide analogue subsequent to incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide, but which may no longer be present after introduction of an additional nucleotide or nucleotide analogue to the oligonucleotide or oligonucleotide analogue or which may be removed after the synthesis of the entire oligonucleotide or oligonucleotide analogue.

The protecting group may be removed by a number of suitable techniques known to the person skilled in the art, however preferably, the protecting group may be removed by a treatment selected from the group consisting of acid treatment, thiophenol treatment and alkali treatment.

Preferred protecting groups according to the present invention, which may be used to protect the 5' end or the 5' end analogue of a backbone monomer unit may be selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl(DMT).

4, 4'-dimethoxytrityl(DMT) groups may be removed by acid treatment, for example by brief incubation (30 to 60 seconds sufficient) in 3% trichloroacetic acid or in 3% dichloroacetic acid in CH_2Cl_2 .

Preferred protecting groups which may protect a phosphate or phosphoramidite group of a backbone monomer unit may for example be selected from the group

consisting of methyl and 2-cyanoethyl. Methyl protecting groups may for example be removed by treatment with thiophenol or disodium 2-carbamoyl 2-cyanoethylene-1,1-dithiolate. 2-cyanoethyl-groups may be removed by alkali treatment, for example treatment with concentrated aqueous ammonia, a 1:1 mixture of aqueous methylamine and concentrated aqueous ammonia or with ammonia gas.

Intercalator

The term intercalator according to the present invention covers any molecular moiety comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. Preferably an intercalator according to the present invention essentially consists of at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid or nucleic acid analogue.

Preferably, the intercalator comprises a chemical group selected from the group consisting of polyaromates and heteropolyaromates and even more preferably the intercalator essentially consists of a polyaromate or a heteropolyaromate. Most preferably the intercalator is selected from the group consisting of polyaromates and heteropolyaromates.

Polyaromates or heteropolyaromates according to the present invention may consist of any suitable number of rings, such as 1, for example 2, such as 3, for example 4, such as 5, for example 6, such as 7, for example 8, such as more than 8. Furthermore polyaromates or heteropolyaromates may be substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxy and amido.

In one preferred embodiment of the present invention the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of fluorescing.

In another more preferred embodiment of the present invention the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that

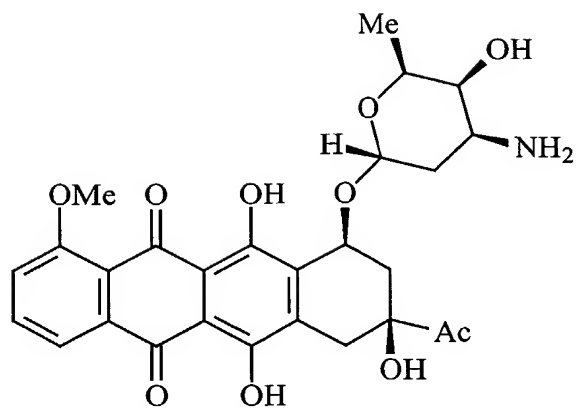
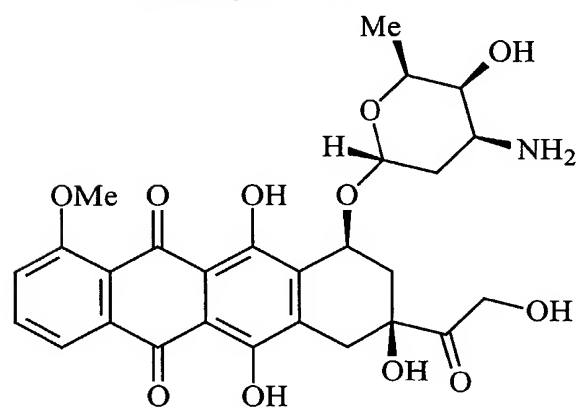
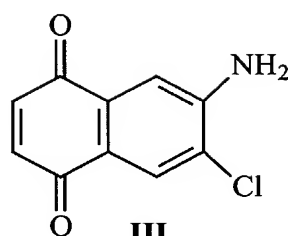
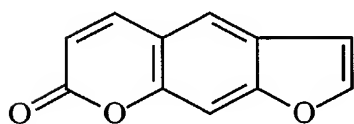
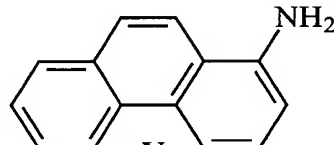
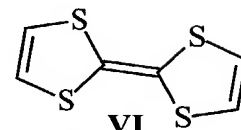
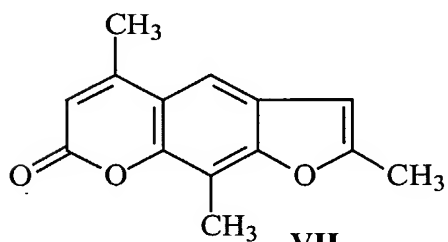
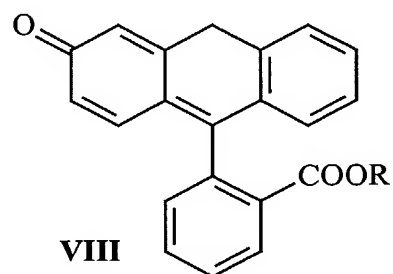
are capable of forming excimers, exciplexes, fluorescence resonance energy transfer (FRET) or charged transfer complexes.

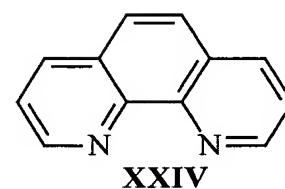
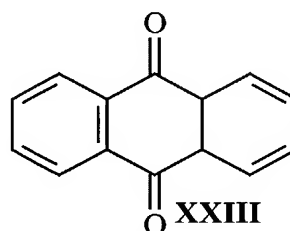
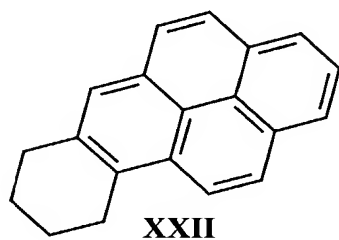
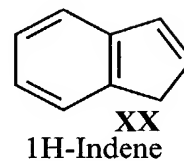
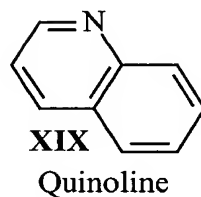
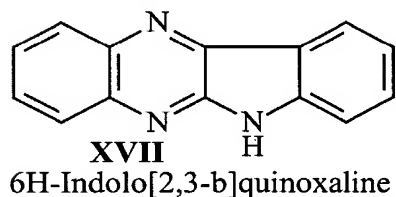
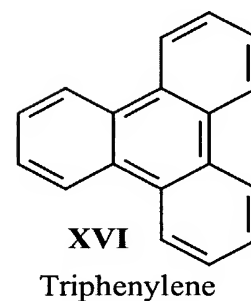
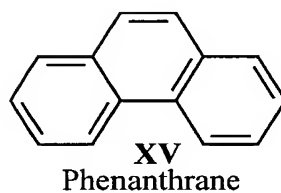
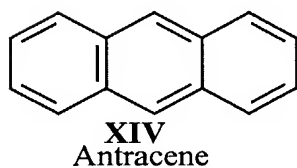
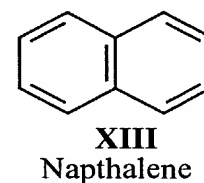
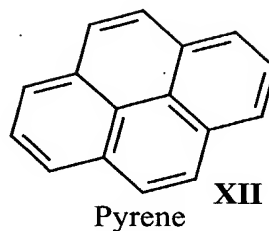
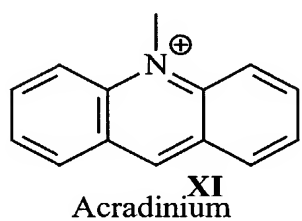
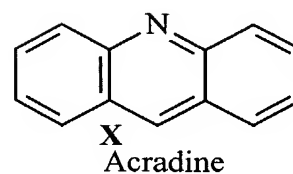
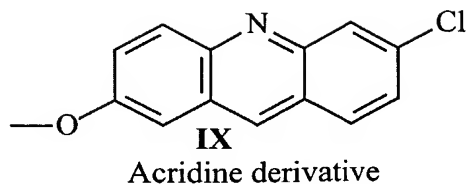
Accordingly, the intercalator may preferably be selected from the group consisting of
5 phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins, psoralens and any of the aforementioned intercalators substituted with one or more selected from
10 the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and/or amido.

Preferably, the intercalator is selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene,
15 phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.

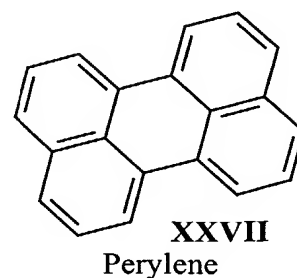
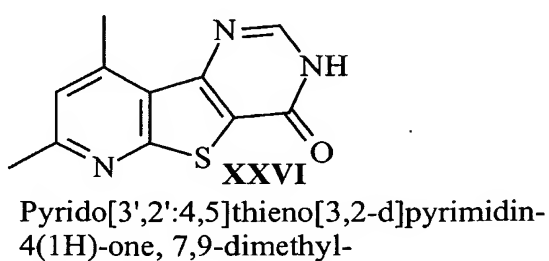
More preferably the intercalator may be selected from the group of intercalators comprising one of the structures as indicated herein below:

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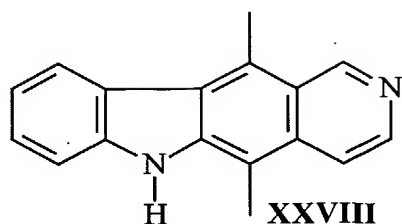
Daunomycin **I**Doxorubicin **II**Naphthoquinone **III**Psoralene **IV**Phenanthroline **V**1,3-Dithiole,
2-(1,3-dithiol-2-ylidene)-
VITrimethylpsoralene **VII**Fluorescein derivative **VIII**



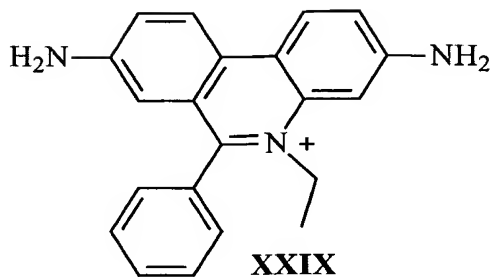
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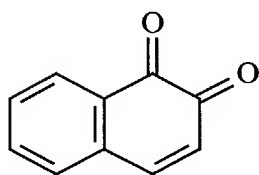
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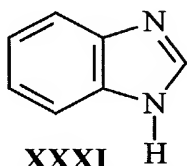
6H-Pyrido[4,3-b]carbazole,
5,11-dimethyl-



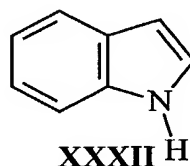
Phenanthridinium,
3,8-diamino-5-ethyl-6-phenyl-



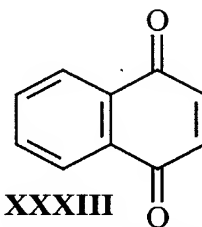
XXX
1,2-Naphthalenedione



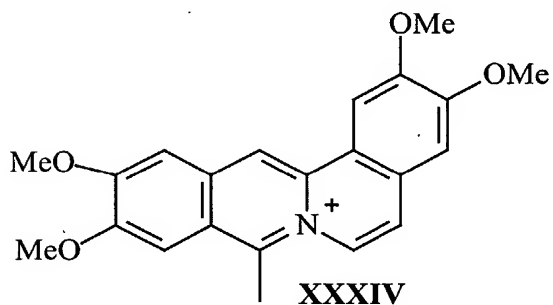
XXXI
1H-Benzimidazole



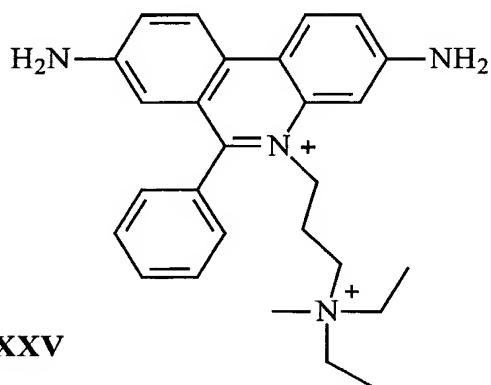
XXXII
1H-Indole



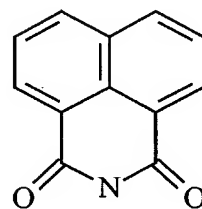
XXXIII
1,4-Naphthalenedione



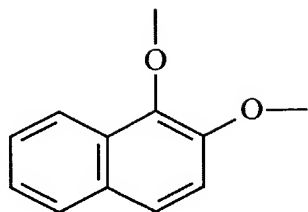
XXXIV
Dibenzo[a,g]quinolizinium,
2,3,10,11-tetramethoxy-8-methyl-



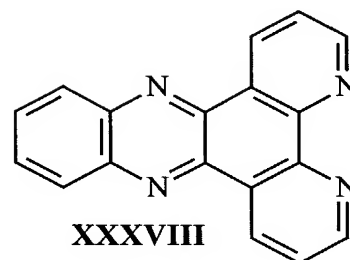
XXXV
Phenanthridinium, 3,8-diamino-5-
[3-(diethylmethylammonio)propyl]-6-phenyl-



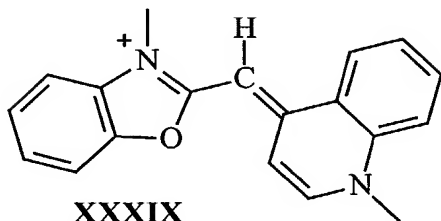
XXXVI
1H-Benz[de]isoquinoline-
1,3(2H)-dione

**XXXVII**

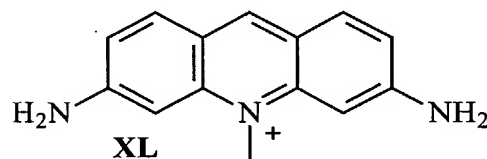
Naphthalene, 1,2-dimethoxy-

**XXXVIII**

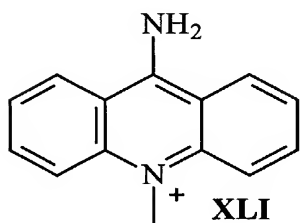
Dipyrido[3,2-a:2',3'-c]phenazine

**XXXIX**

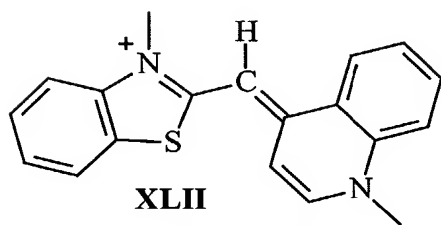
Quinolinium, 4-[(3-ethyl-2(3H)-benzoxazolylidene)methyl]-1-methyl-

**XL**

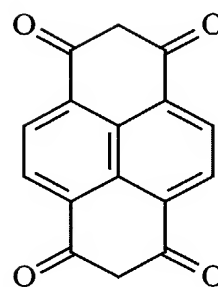
Acridine, 6-amino-3,10-dihydro-3-imino-10-methyl-

**XLI**

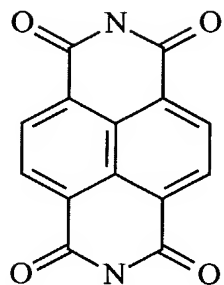
Acridinium, 9-amino-10-methyl-

**XLII**

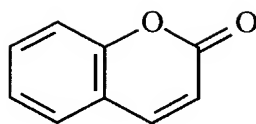
Quinolinium, 1-methyl-4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]-

**XLIII**

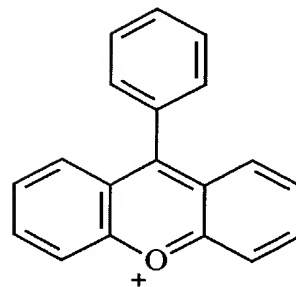
1,3,6,8(2H,7H)-Pyrenetetrone

**XLIV**

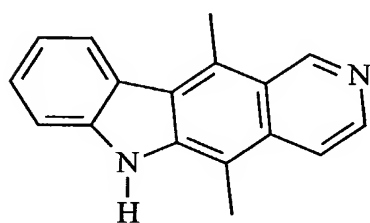
Benzo[lmn][3,8]phenanthroline-
1,3,6,8(2H,7H)-tetrone

**XLV**

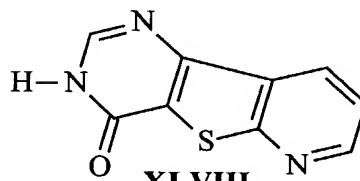
2H-1-Benzopyran-2-one

**XLVI**

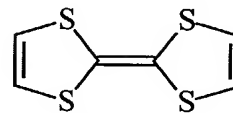
Xanthylium, 9-phenyl-

**XLVII**

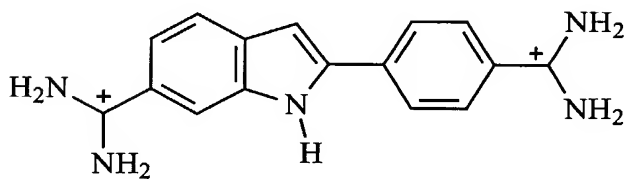
Ellipticine

**XLVIII**

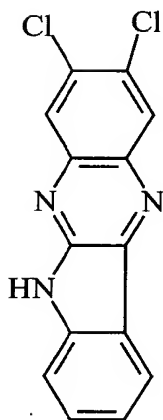
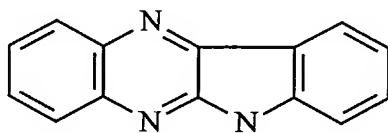
Pyrido[3',2':4,5]thieno
[3,2-d]pyrimidin-
4(1H)-one

**XLIX**

Fulvalene

**L**

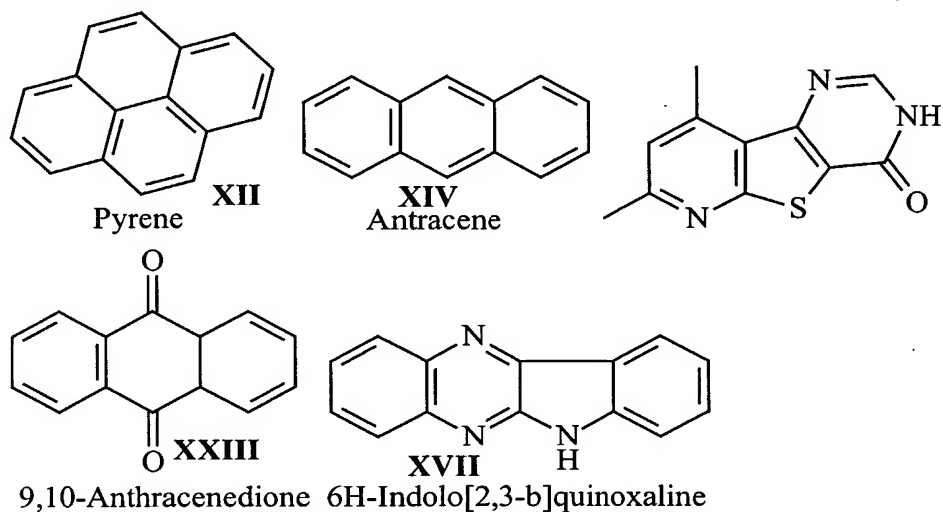
DAPI

**LI****LII**

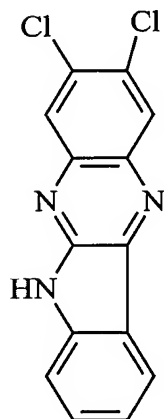
Even more preferably the intercalator may be selected from the group of intercalators comprising one of the intercalator structures above numbered V, XII, XIV, XV, XVII, XXIII, XXVI, XXVIII, XLVII, LI and LII as well as derivatives thereof.

5

Most preferably the intercalator is selected from the group of intercalator structures above numbered XII, XIV, XVII, XXIII, LI.



10



as well as derivatives thereof

15

The above list of examples is not to be understood as limiting in any way, but only as to provide examples of possible structures for use as intercalators. In addition, the substitution of one or more chemical groups on each intercalator to obtain modified structures is also included in the present invention.

The intercalator moiety of the intercalator pseudonucleotide is linked to the backbone unit by the linker. When going from the backbone along the linker to the intercalating moiety, the linker and intercalator connection is defined as the bond between a linker atom and the first atom being part of a conjugated system that is able to co-stack with nucleobases of a strand of an oligonucleotide or oligonucleotide analogue when said oligonucleotide or oligonucleotide analogue is hybridised to an oligonucleotide analogue comprising said intercalator pseudonucleotide.

In one embodiment of the present invention, the linker may comprise a conjugated system and the intercalator may comprise another conjugated system. In this case the linker conjugated system is not capable of costacking with nucleobases of said opposite oligonucleotide or oligonucleotide analogue strand.

Linker

The linker of an intercalator pseudonucleotide according to the present invention is a moiety connecting the intercalator and the backbone monomer of said intercalator pseudonucleotide. The linker may comprise one or more atom(s) or bond(s) between atoms.

By the definitions of backbone and intercalating moieties defined herein above, the linker is the shortest path linking the backbone and the intercalator. If the intercalator is linked directly to the backbone, the linker is a bond.

The linker usually consists of a chain of atoms or a branched chain of atoms. Chains can be saturated as well as unsaturated. The linker may also be a ring structure with or without conjugated bonds.

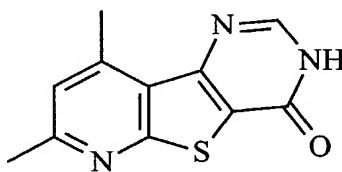
For example the linker may comprise a chain of m atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

In some embodiments the total length of the linker and the intercalator of the intercalator pseudonucleotides according to the present invention preferably is between 8 and 13 Å (see herein below). Accordingly, m should be selected dependent on the size of the intercalator of the specific intercalator pseudonucleotide.

I.e. m should be relatively large, when the intercalator is small and m should be relatively small when the intercalator is large. For most purposes however m will be an integer from 1 to 7, such as from 1-6, such as from 1-5, such as from 1-4. As described above the linker may be an unsaturated chain or another system involving conjugated bonds. For example the linker may comprise cyclic conjugated structures. Preferably, m is from 1 to 4 when the linker is an saturated chain.

When the intercalator is pyrene, m is preferably an integer from 1 to 7, such as from 1-6, such as from 1-5, such as from 1-4, more preferably from 1 to 4, even more preferably from 1 to 3, most preferably m is 2 or 3.

When the intercalator has the structure



m is preferably from 2 to 6, more preferably 2.

The chain of the linker may be substituted with one or more atoms selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

In one embodiment the linker is an azaalkyl, oxaalkyl, thiaalkyl or alkyl chain. For example the linker may be an alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N, P, Se, Si, Ge, Sn and Pb. In a preferred embodiment the linker consists of an unbranched alkyl chain, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit and wherein each C is substituted with 2 H. More preferably, said unbranched alkyl chain is from 1 to 5 atoms long, such as from 1 to 4 atoms long, such as from 1 to 3 atoms long, such as from 2 to 3 atoms long.

5 In another embodiment of the invention the linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb. For example the linker may be such a ring structure substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

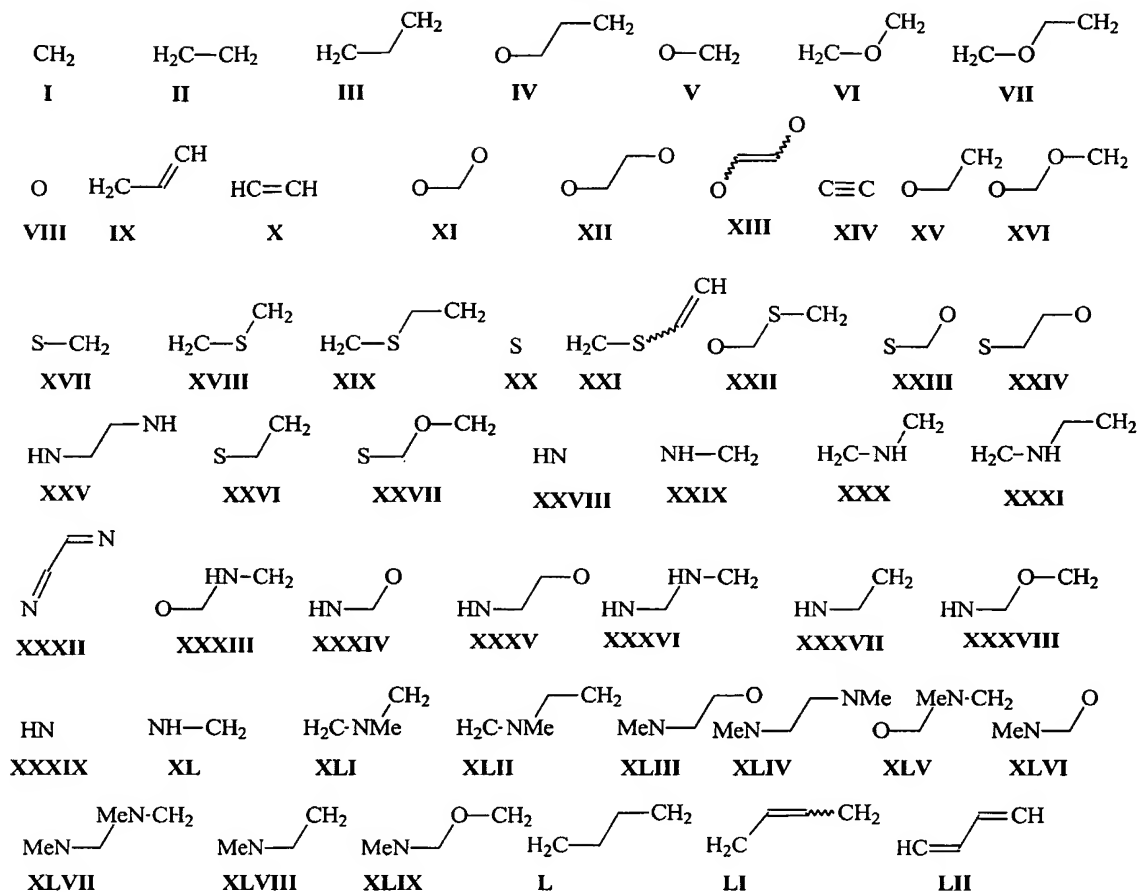
10 In another embodiment the linker consists of from 1-6 C atoms, from 0-3 of each of the following atoms O, S, N. More preferably the linker consists of from 1-6 C atoms and from 0-1 of each of the atoms O, S, N.

15 In a preferred embodiment the linker consists of a chain of C, O, S and N atoms, optionally substituted. Preferably said chain should consist of at the most 3 atoms, thus comprising from 0 to 3 atoms selected individually from C, O, S, N, optionally substituted.

20 In a preferred embodiment the linker consists of a chain of C, N, S and O atoms, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

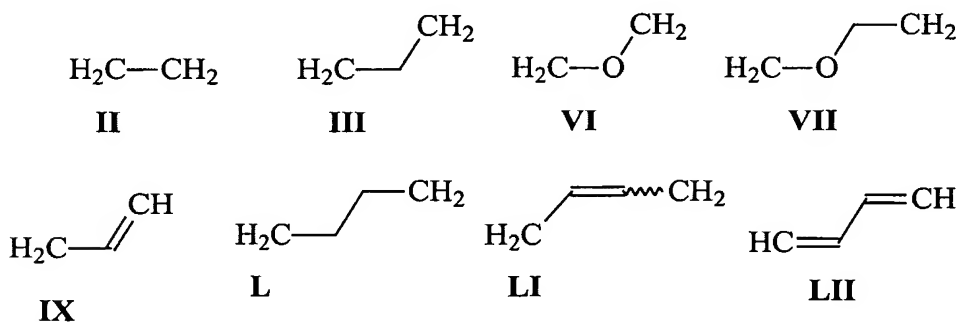
25 Preferably such a chain comprise one of the linkers shown below, most preferably the linker consist of one of the molecule shown below:

55



In a preferred embodiment the chain comprise one of the linkers shown below, more preferably the linker consist of one of the molecule shown below:

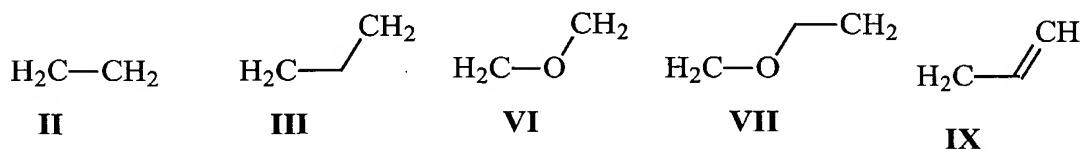
5



10

In a more preferred embodiment the chain comprise one of th linkers shown below, more preferably the linker consist of one of the molecule shown below:

In a more preferred embodiment the chain comprise one of th linkers shown below, more preferably the linker consist of one of the molecule shown below:



5

The linker constitutes Y in the formula for the intercalator pseudonucleotide X-Y-Q, as defined above, and hence X and Q are not part of the linker.

Intercalator pseudonucleotides

10 Intercalator pseudonucleotides according to the present invention preferably have the general structure



15 wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,

20 Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

25

wherein the total length of Q and Y is in the range from 7 Å to 20 Å,

with the proviso that when the intercalator is pyrene the total length of Q and Y is in the range from 9 Å to 13 Å.

30

Furthermore, in a preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit, wherein said backbone

monomer unit is capable of being incorporated into the phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 4 atoms are separating the two phosphor atoms of the backbone that are closest to the intercalator.

5

The intercalator pseudonucleotides preferably do not comprise a nucleobase capable of forming Watson-Crick hydrogen bonding. Hence intercalator pseudonucleotides according to the invention are preferably not capable of Watson-Crick base pairing.

10

Preferably, the total length of Q and Y is in the range from 7 Å to 20 Å, more preferably, from 8 Å to 15 Å, even more preferably from 8 Å to 13 Å, even more preferably from 8.4 Å to 12 Å, most preferably from 8.59 Å to 10 Å or from 8.4 Å to 10.5 Å.

15

When the intercalator is pyrene the total length of Q and Y is preferably in the range of 8 Å to 13 Å, such as from 9 Å to 13 Å, more preferably from 9.05 Å to 11 Å, such as from 9.0 Å to 11 Å, even more preferably from 9.05 to 10 Å, such as from 9.0 to 10 Å, most preferably about 9.8 Å.

20

The total length of the linker (Y) and the intercalator (Q) should be determined by determining the distance from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit. Preferably, the distance should be the maximal distance in which bonding angles and normal chemical laws are not broken or distorted in any way.

25

The distance should preferably be determined by calculating the structure of the free intercalating pseudonucleotide with the lowest conformational energy level, and then determining the maximum distance that is possible from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit without bending, stretching or otherwise distorting the structure more than simple rotation of bonds

35

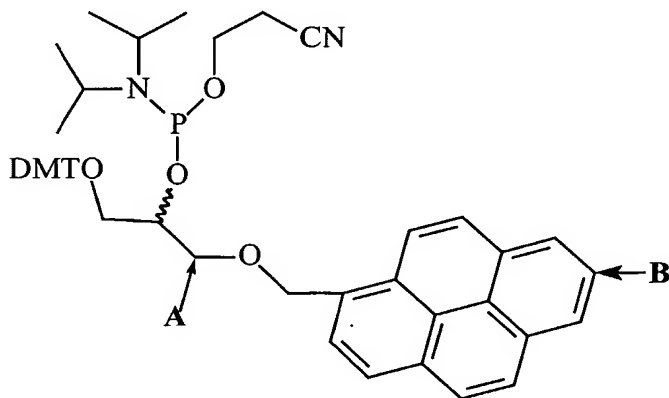
that are free to rotate (e.g. not double bonds or bonds participating in a ring structure).

Preferably the energetically favorable structure is found by *ab initio* or forcefields calculations.

Even more preferably the distance should be determined by a method consisting of the following steps:

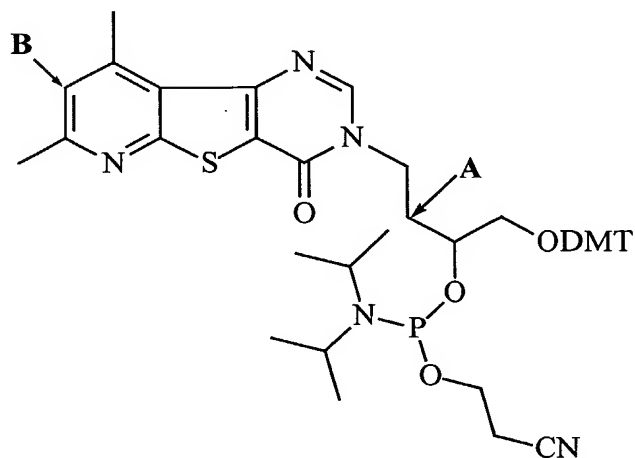
- a) the structure of the intercalator pseudonucleotide of interest is drawn by computer using the programme ChemWindow® 6.0 (BioRad); and
- b) the structure is transferred to the computer programme SymApps™ (BioRad); and
- c) the 3-dimensional structure comprising calculated lengths of bonds and bonding angles of the intercalator pseudonucleotide is calculated using the computer programme SymApps™ (BioRad); and
- d) the 3 dimensional structure is transferred to the computer programme RasWin Molecular Graphics Ver. 2.6-ucb; and
- e) the bonds are rotated using RasWin Molecular Graphics Ver. 2.6-ucb to obtain the maximal distance (the distance as defined herein above); and
- f) the distance is determined.

For example when the intercalator pseudonucleotide has the following structure:



the total length of Q and Y is determined by measuring the linear distance from the center of the atom at A to the center of the atom at B, which in the above example is 9,79 Å.

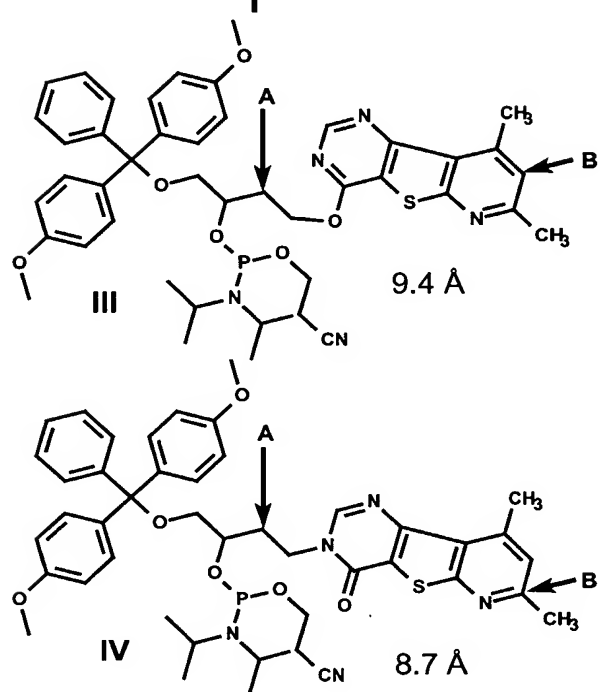
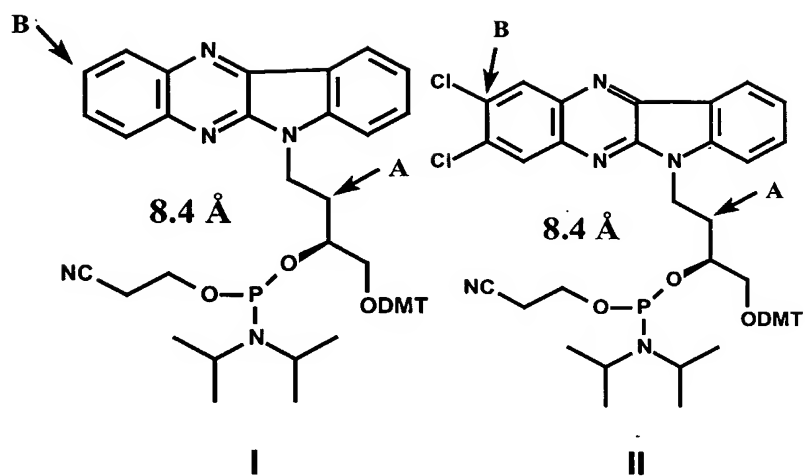
- 5 In another example the intercalator pseudonucleotide has the following structure:



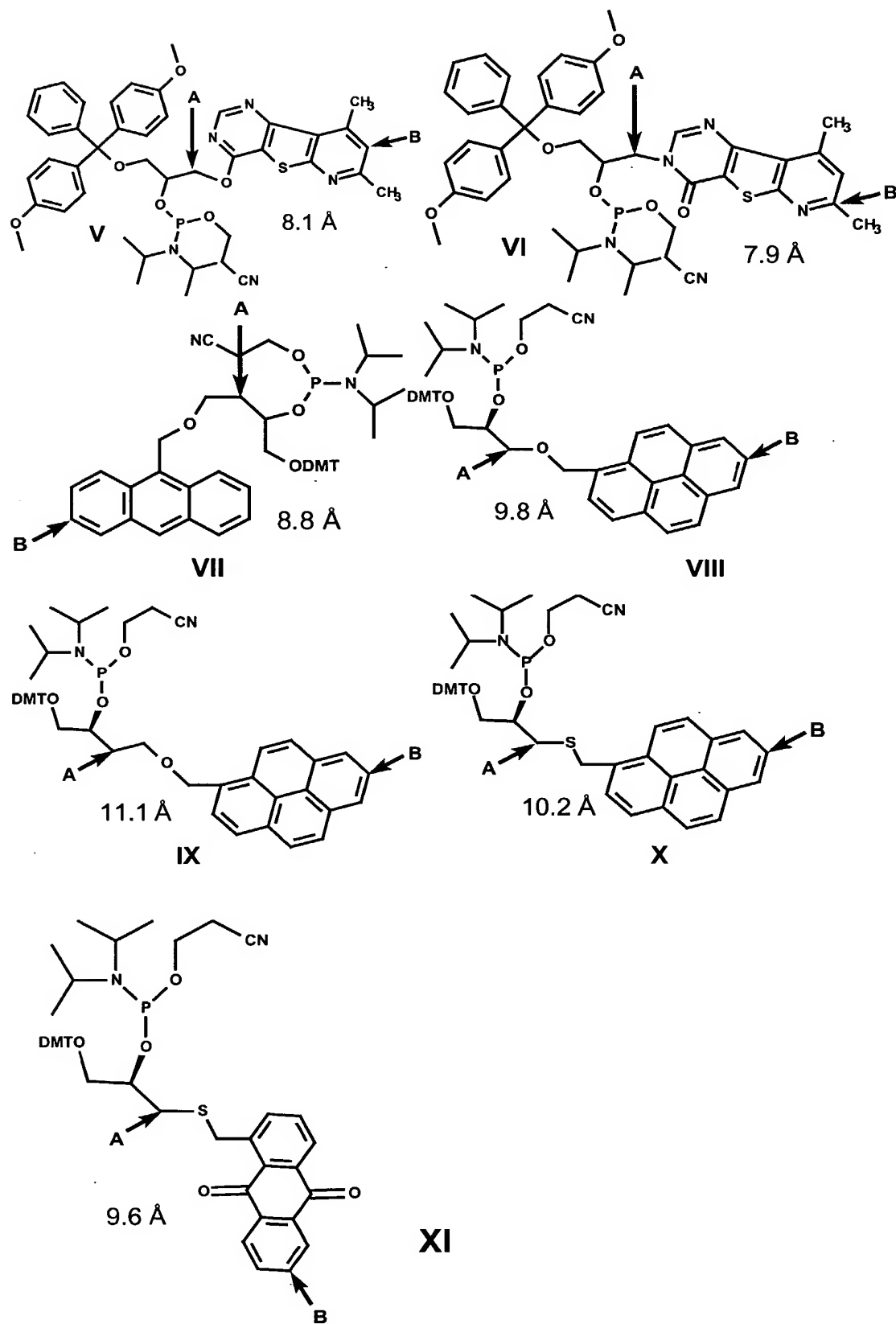
- 10 The total length of Q and Y, which is measured in a straight line from the center of the atom at A to the center of the atom at B is 8.71 Å.

Below here a measure for the length measured in a straight line for a preferred series of intercalator pseudonucleotides is disclosed:

60



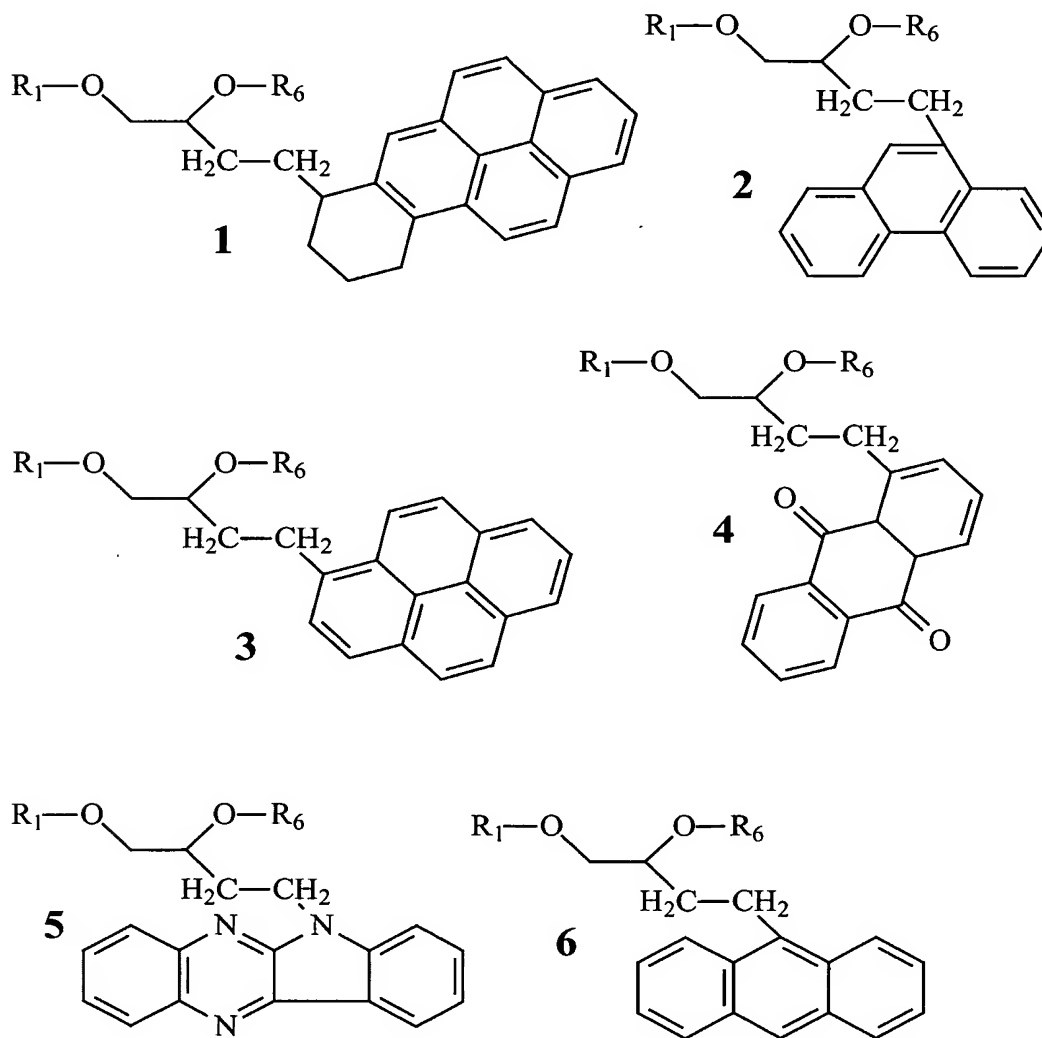
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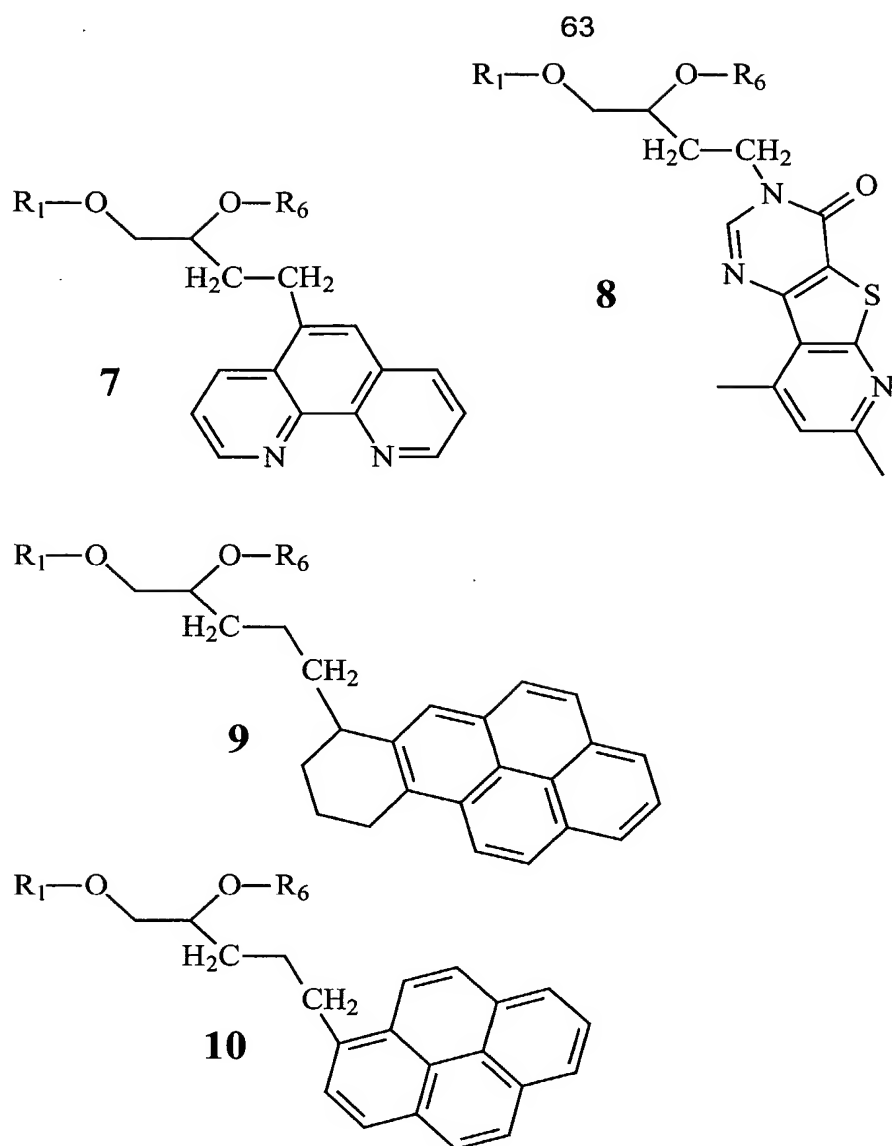


Intercalator pseudonucleotides according to the present invention may be any combination of the above mentioned backbone monomer units, linkers and intercalators.

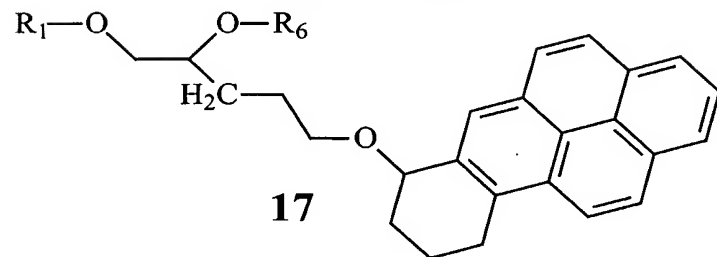
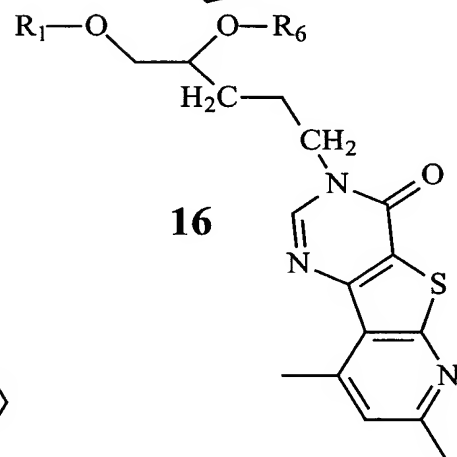
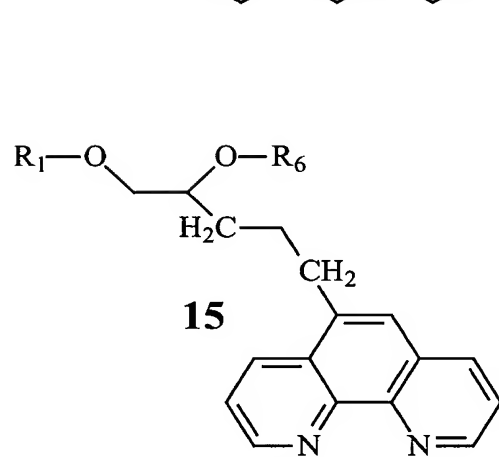
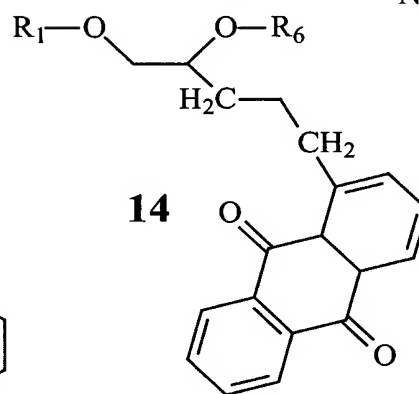
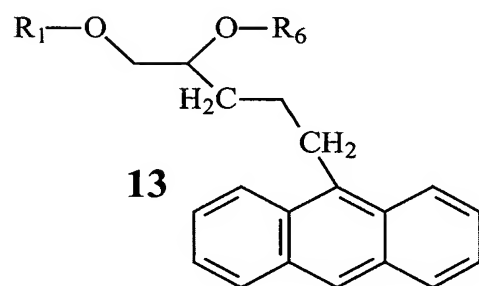
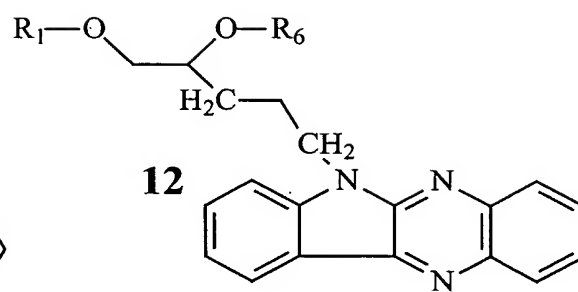
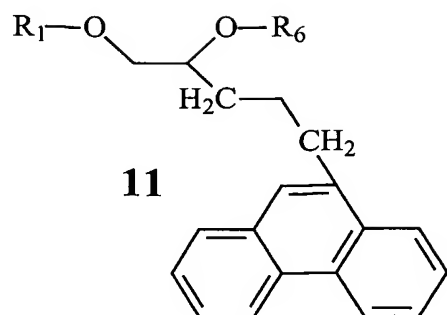
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In one embodiment of the invention the intercalator pseudonucleotide is selected from the group consisting of intercalator pseudonucleotides with the structures 1) to 9 as indicated herein below:

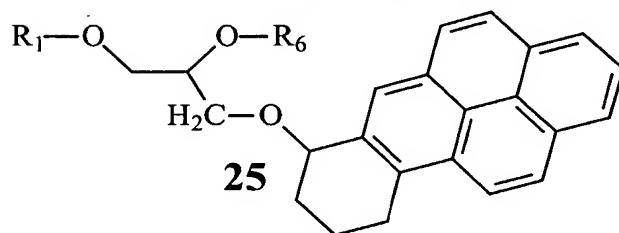
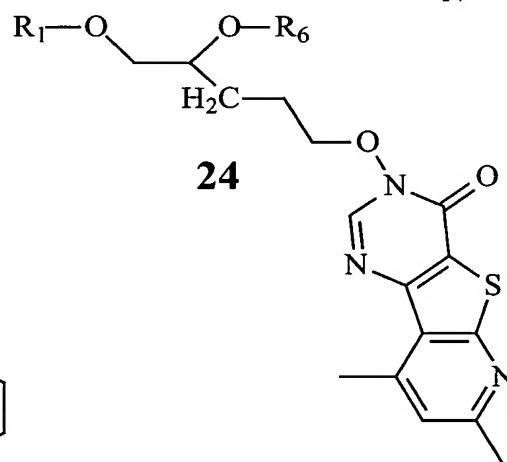
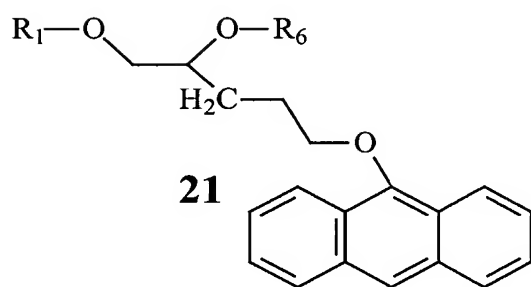
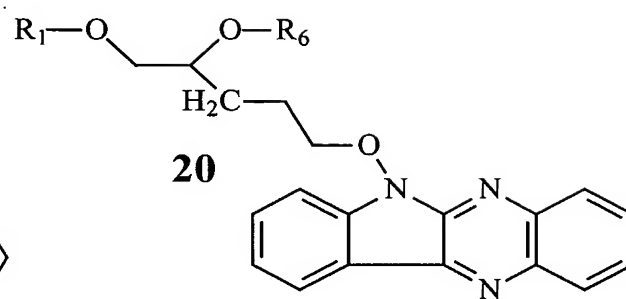
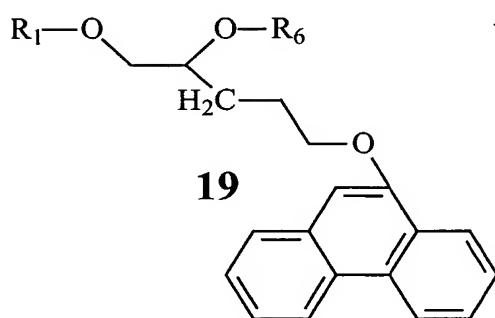
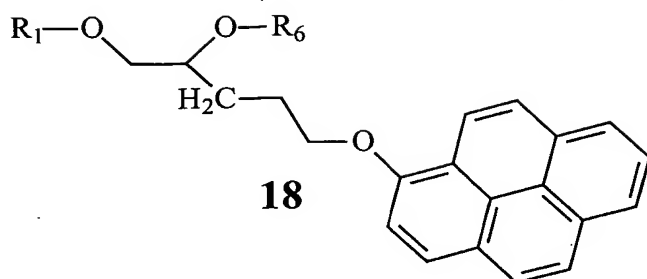


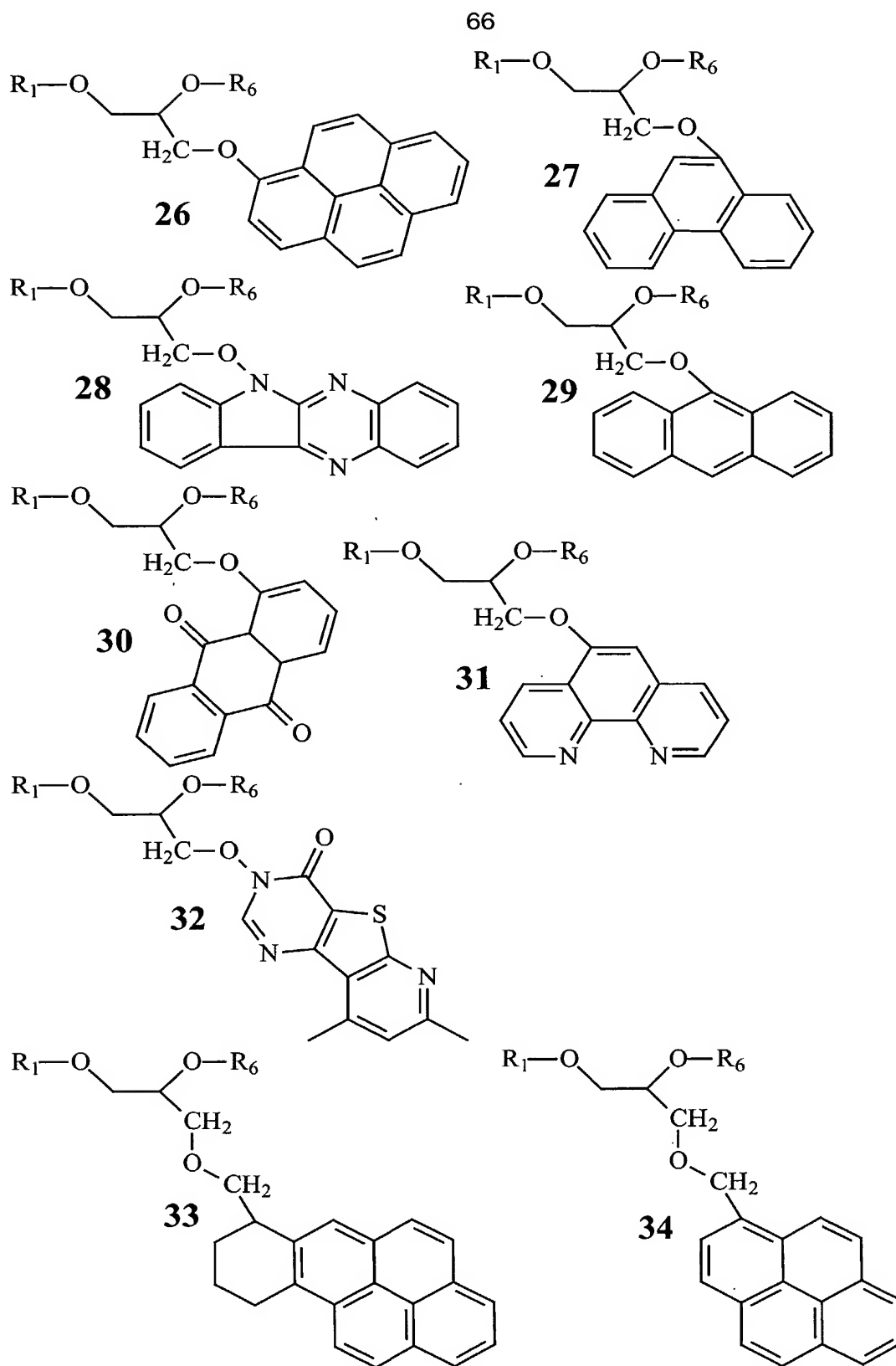


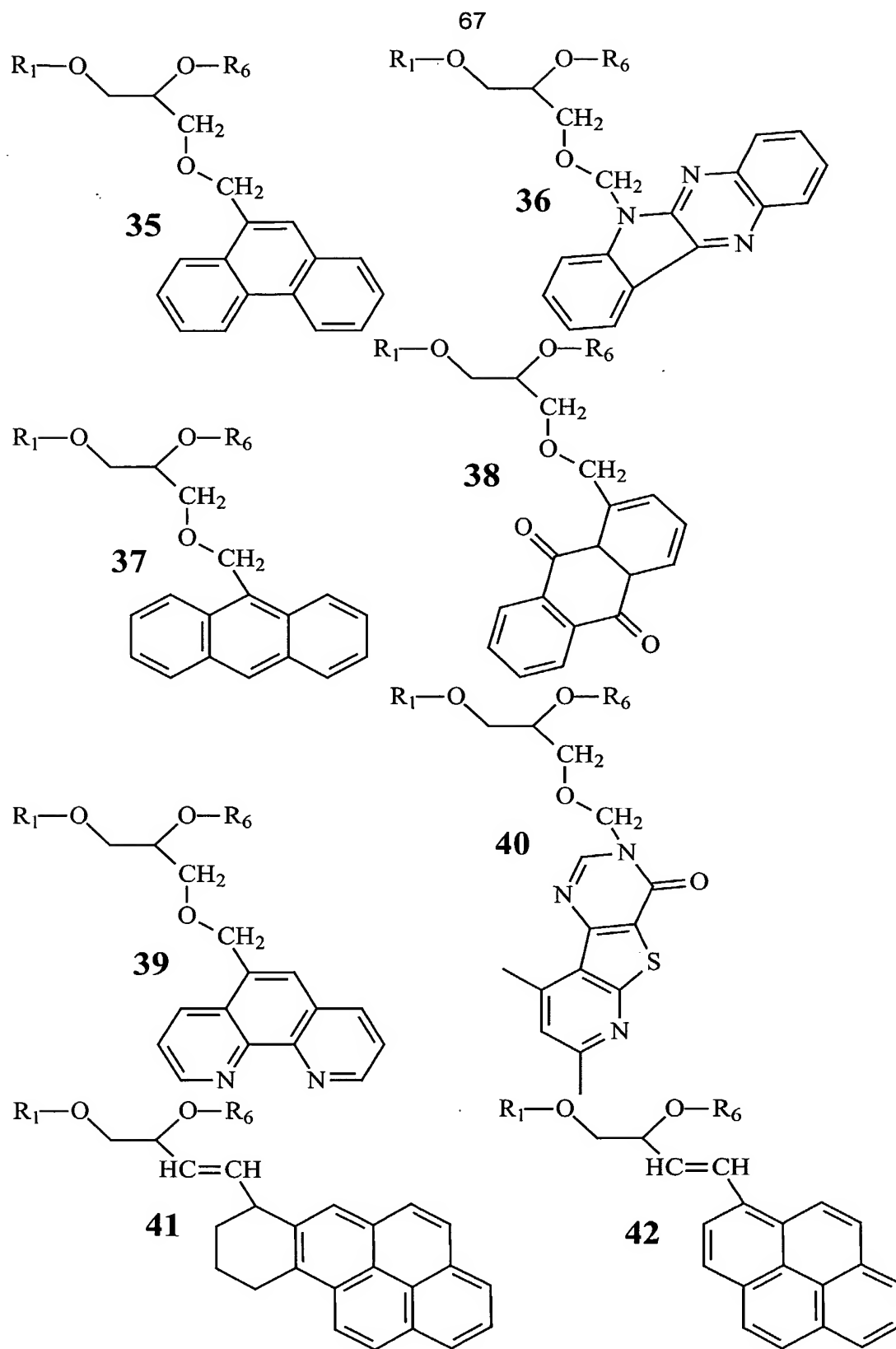
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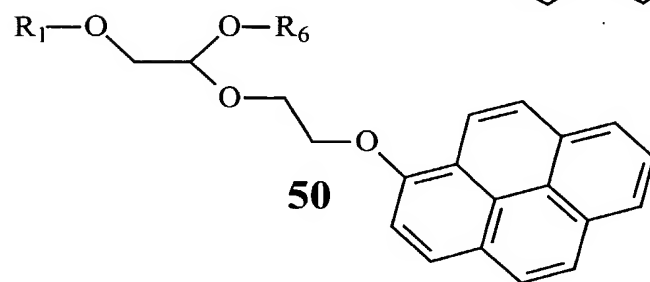
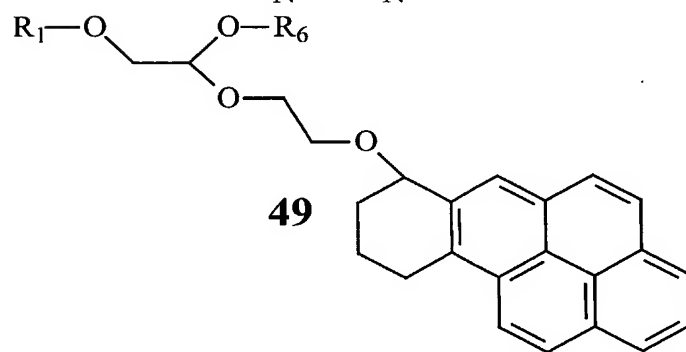
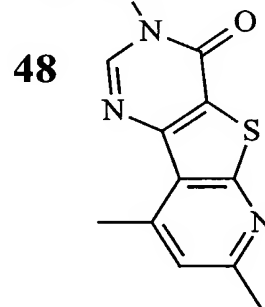
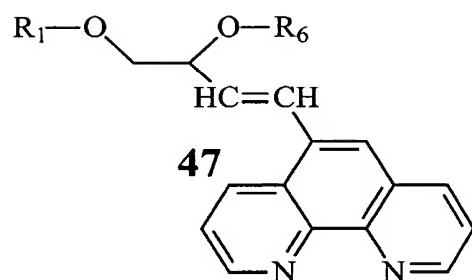
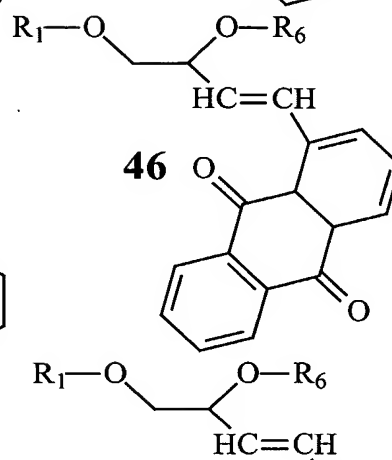
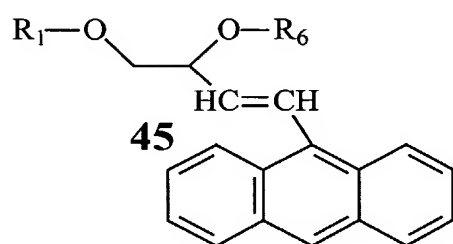
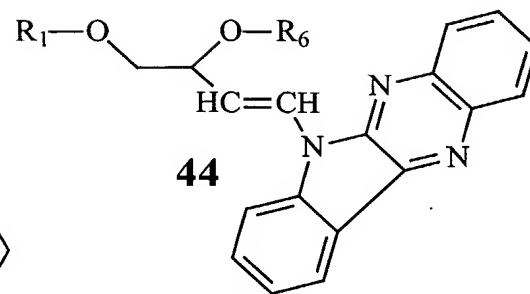
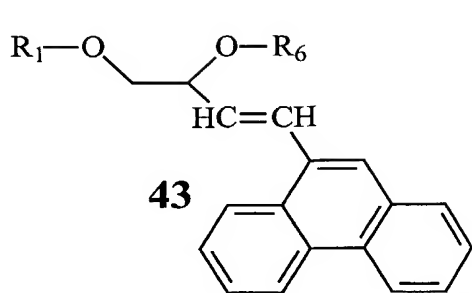
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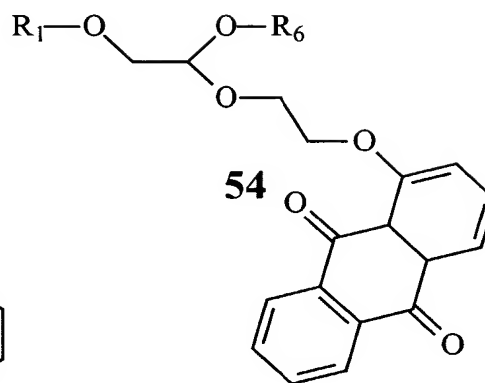
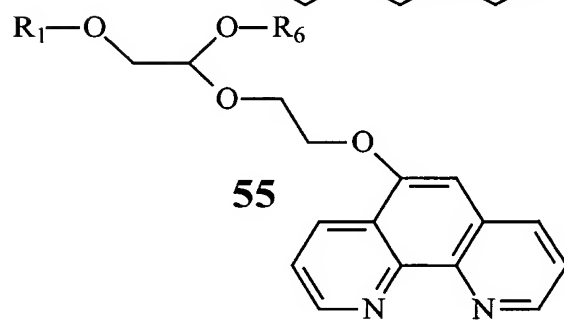
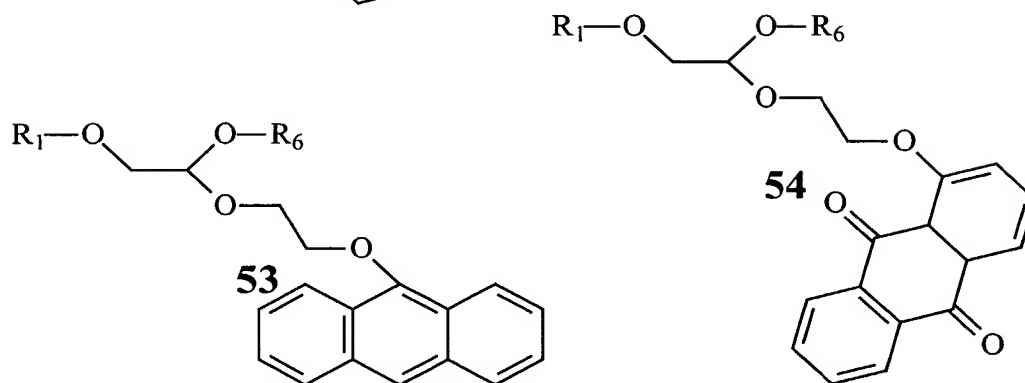
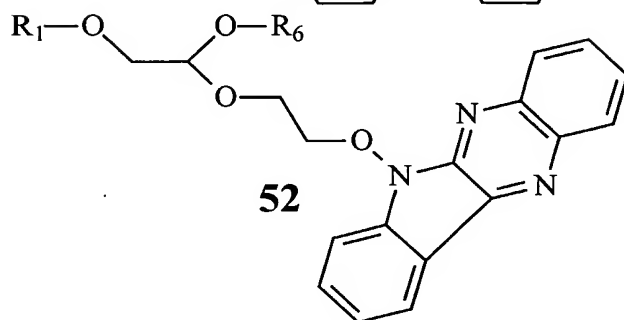
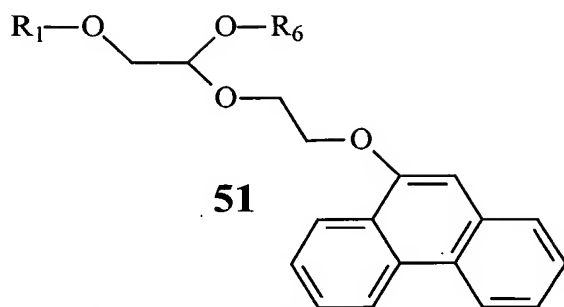




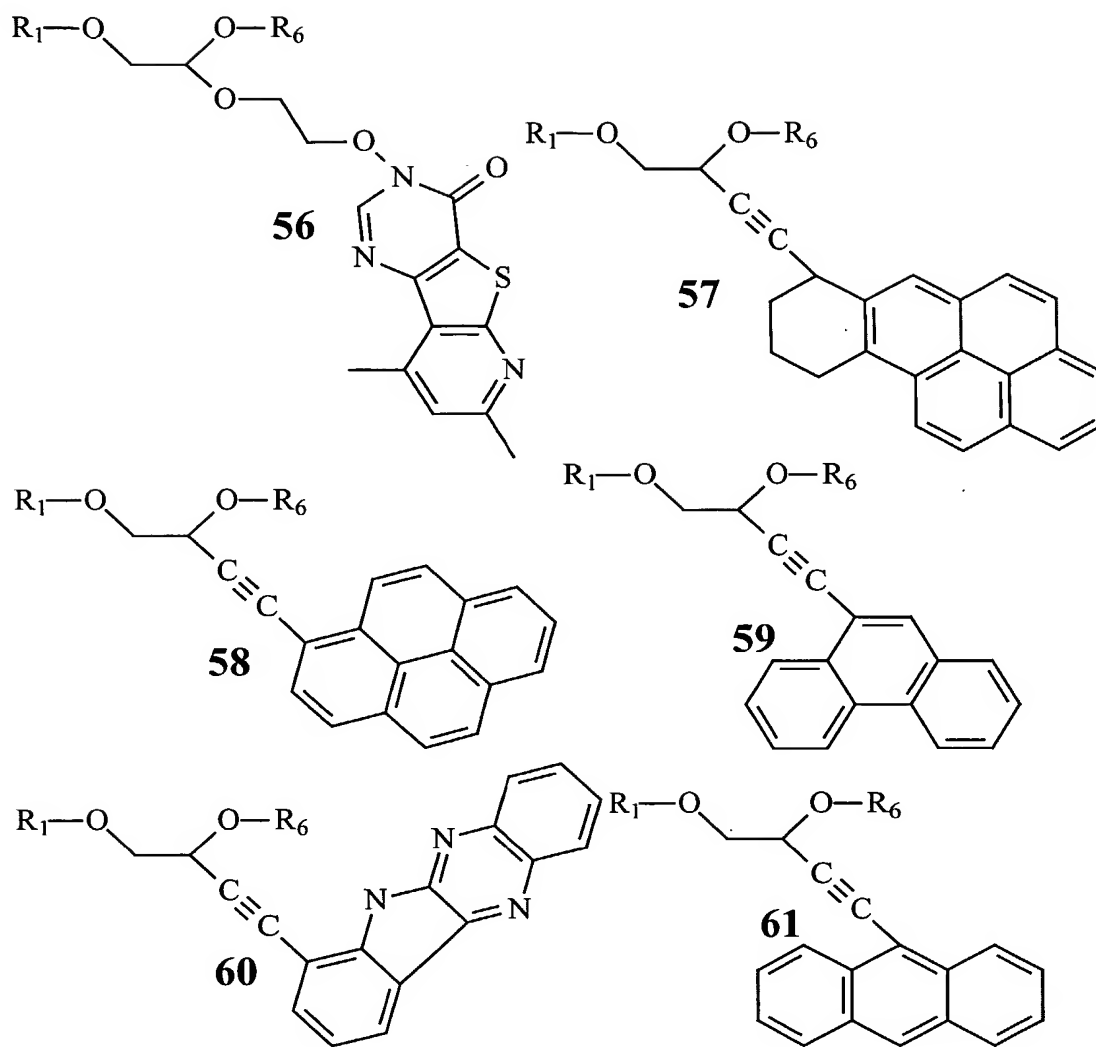


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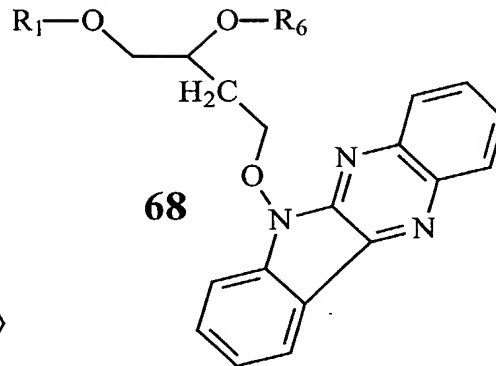
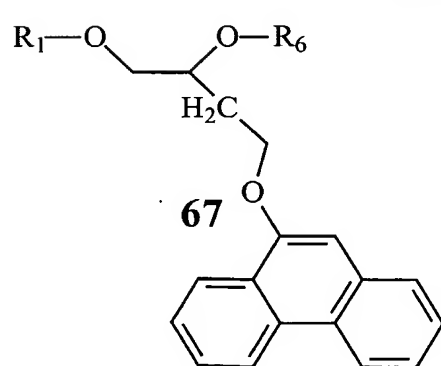
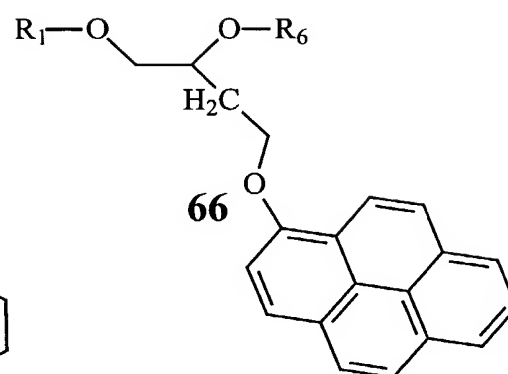
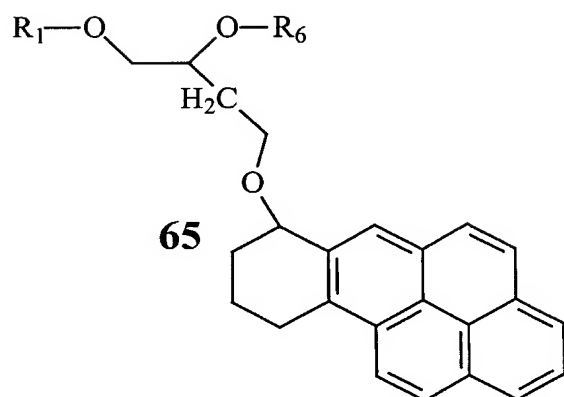
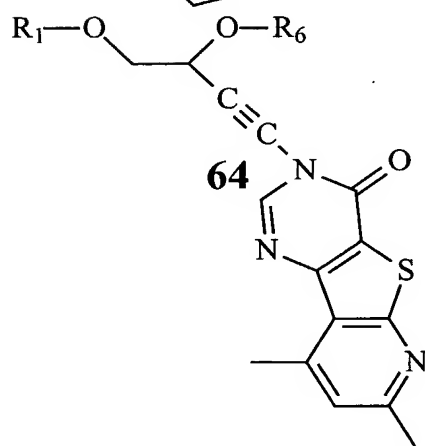
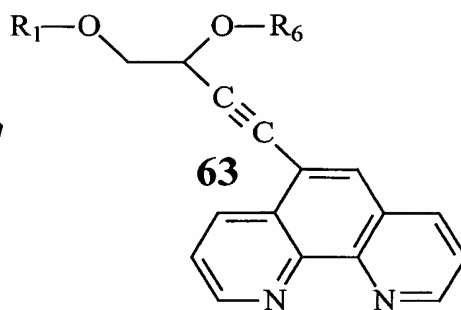
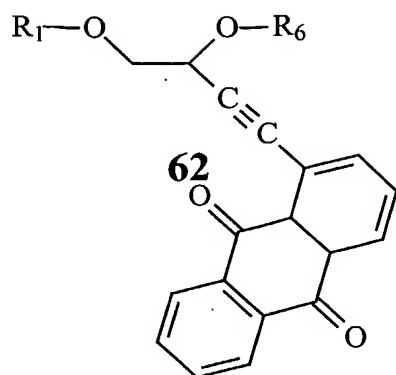


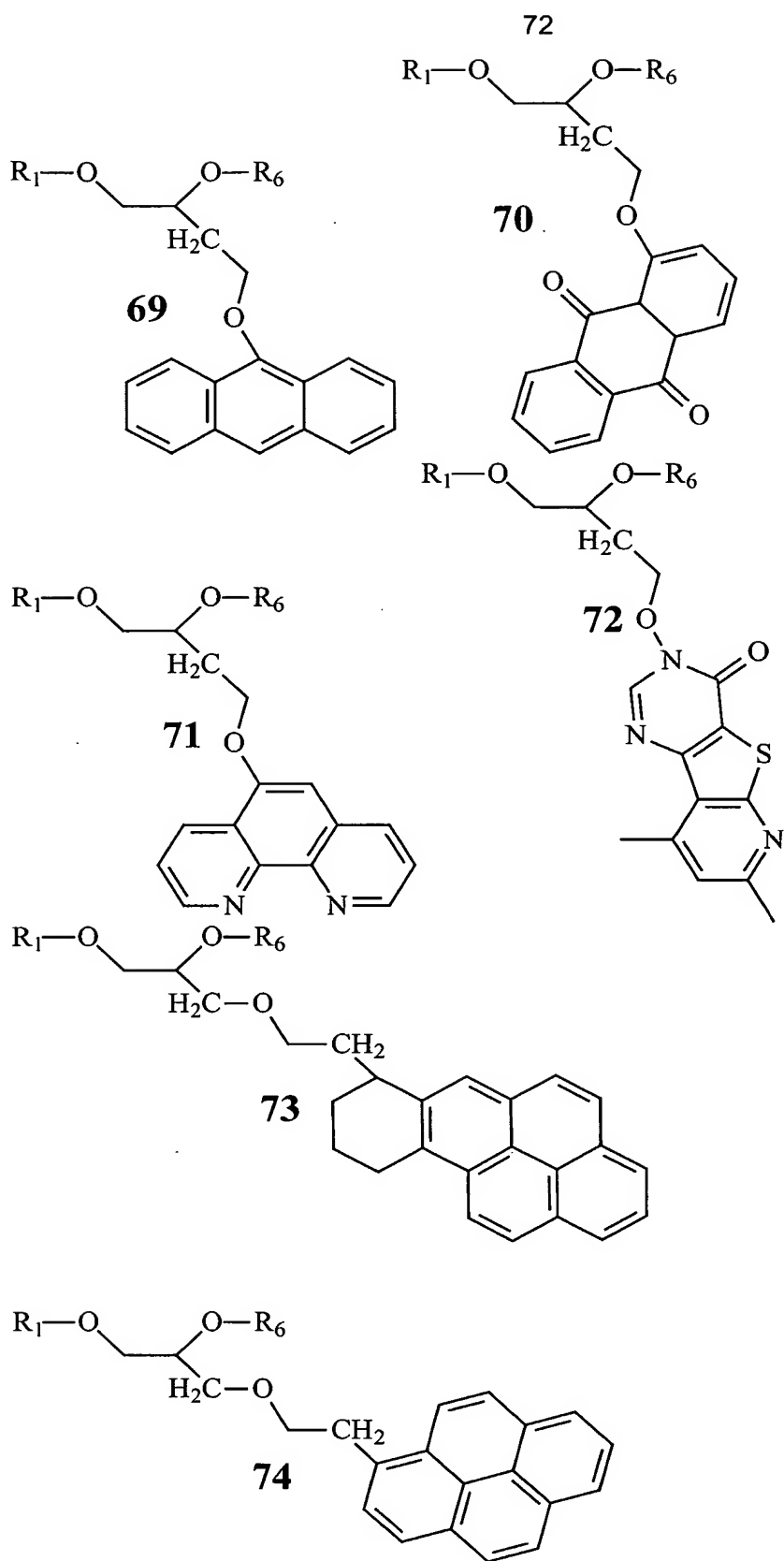


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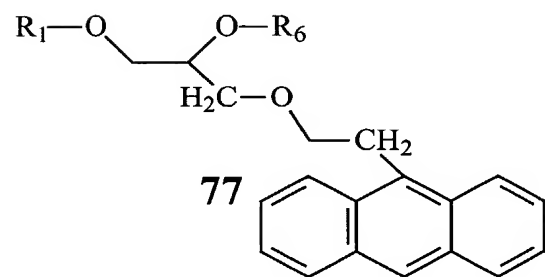
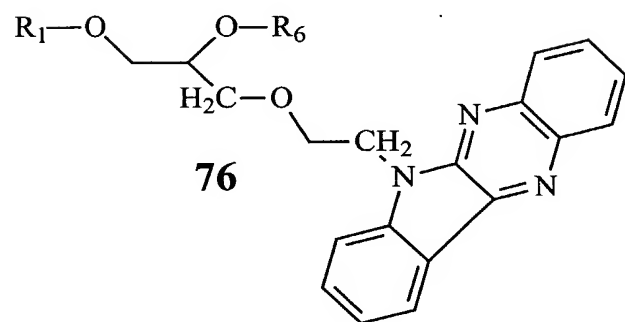
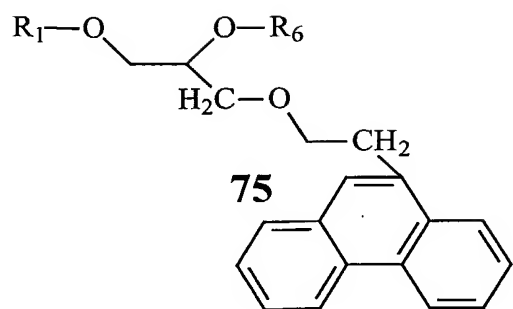


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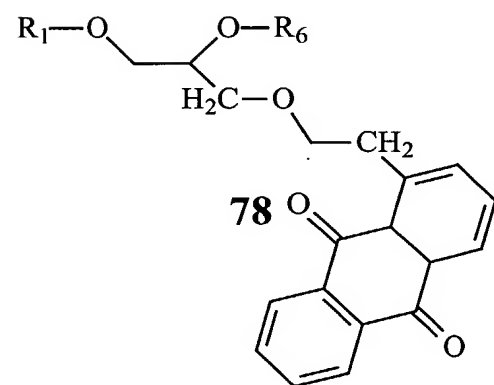




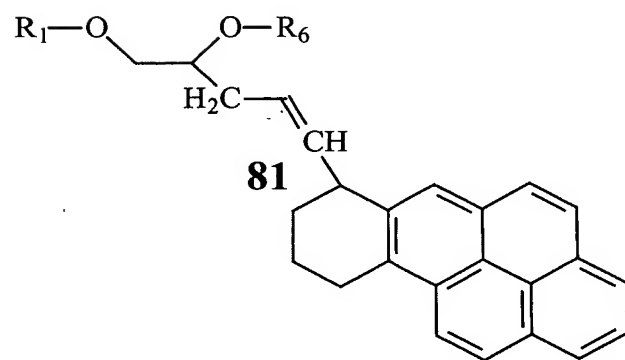
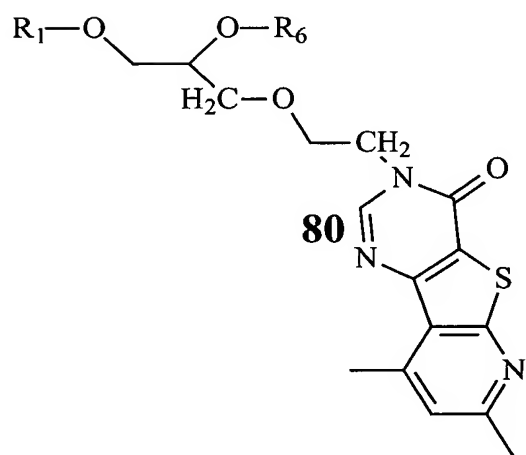
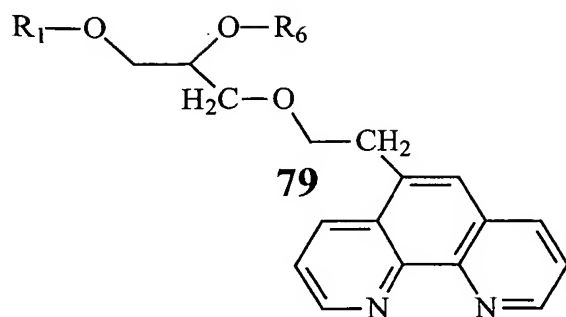
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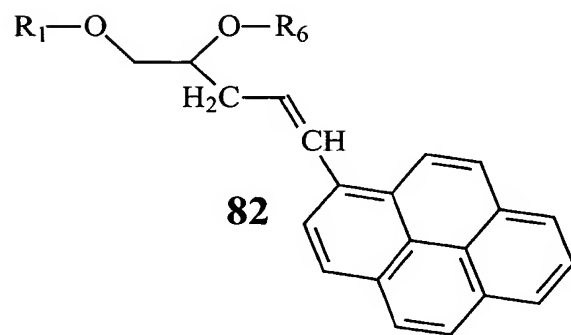
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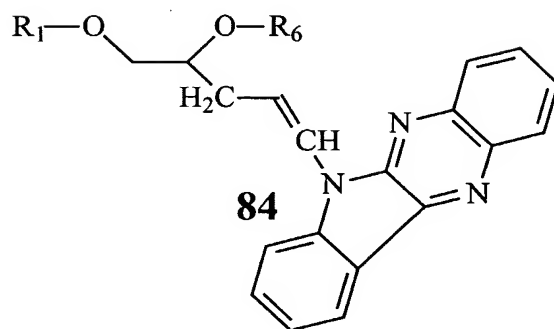
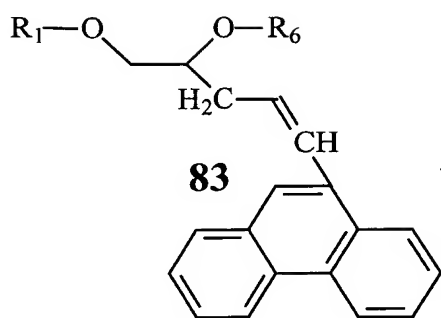
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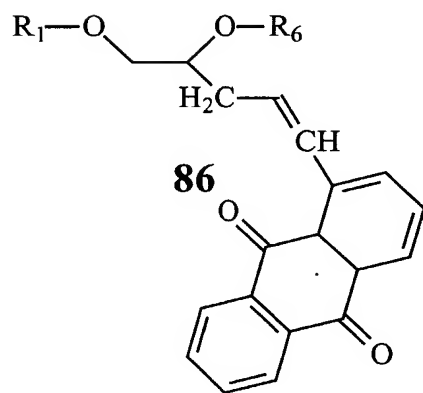
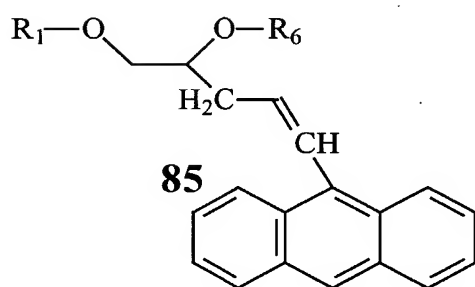
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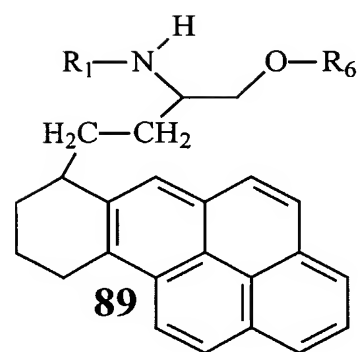
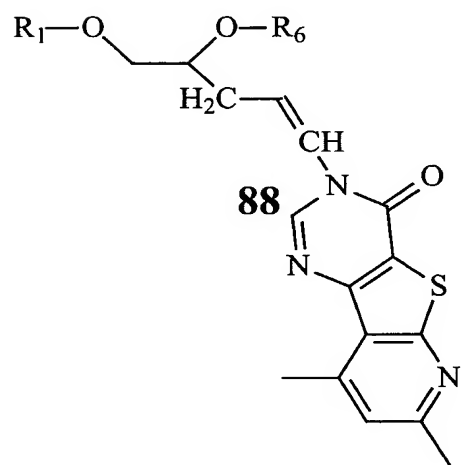
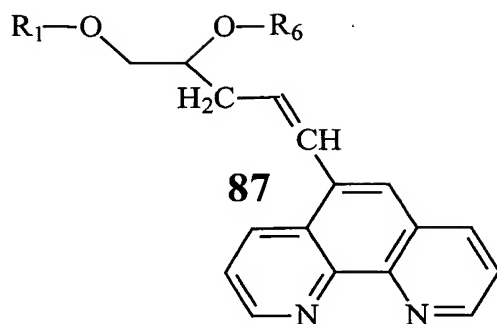


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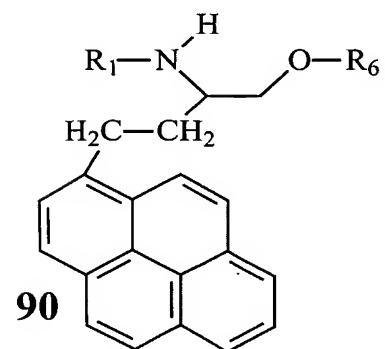


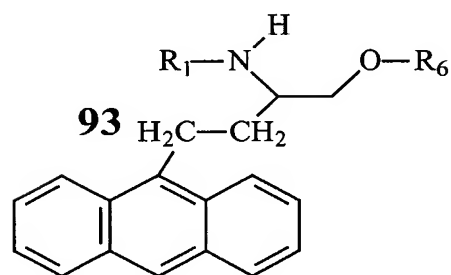
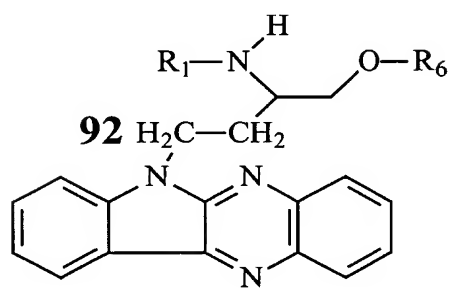
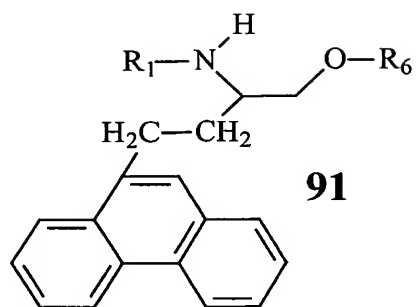
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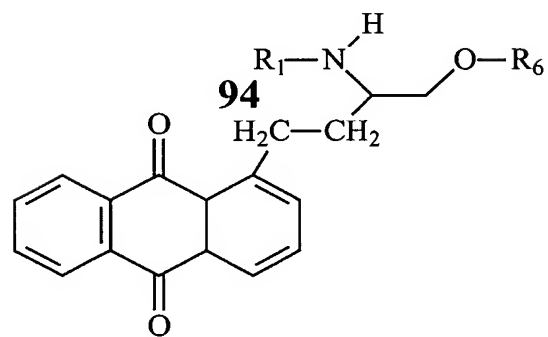


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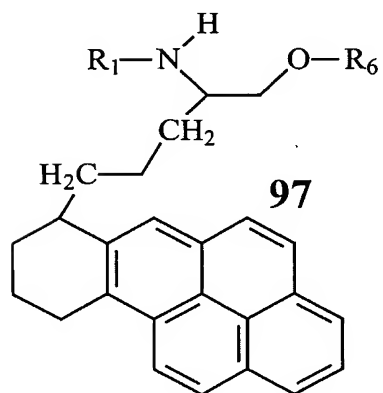
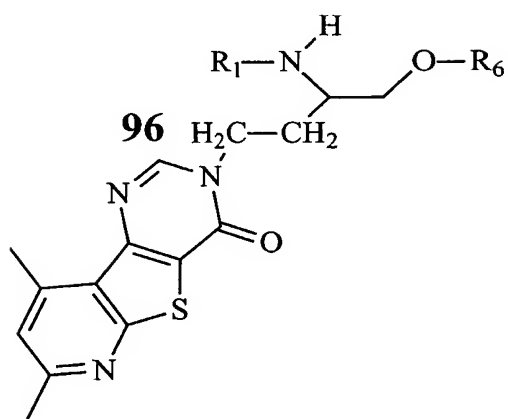
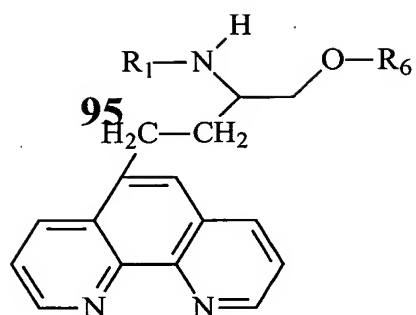


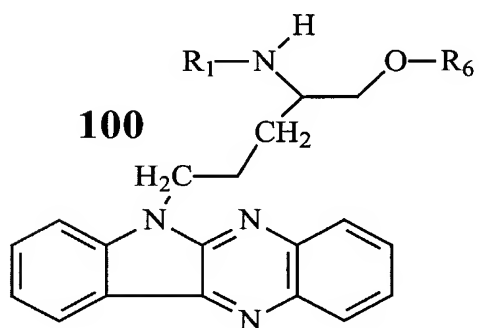
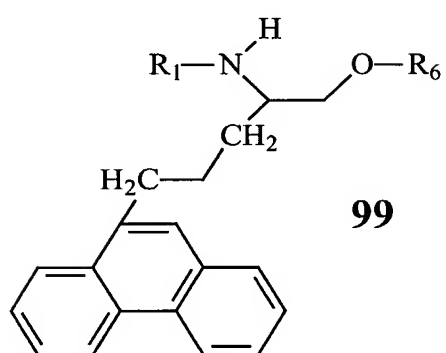
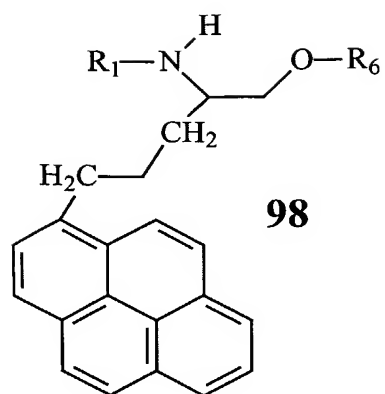


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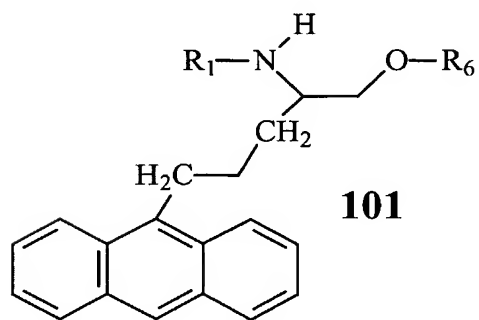


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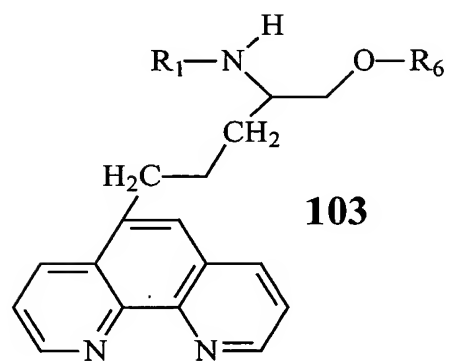
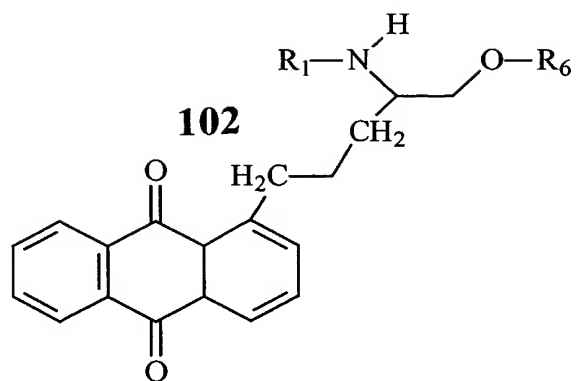




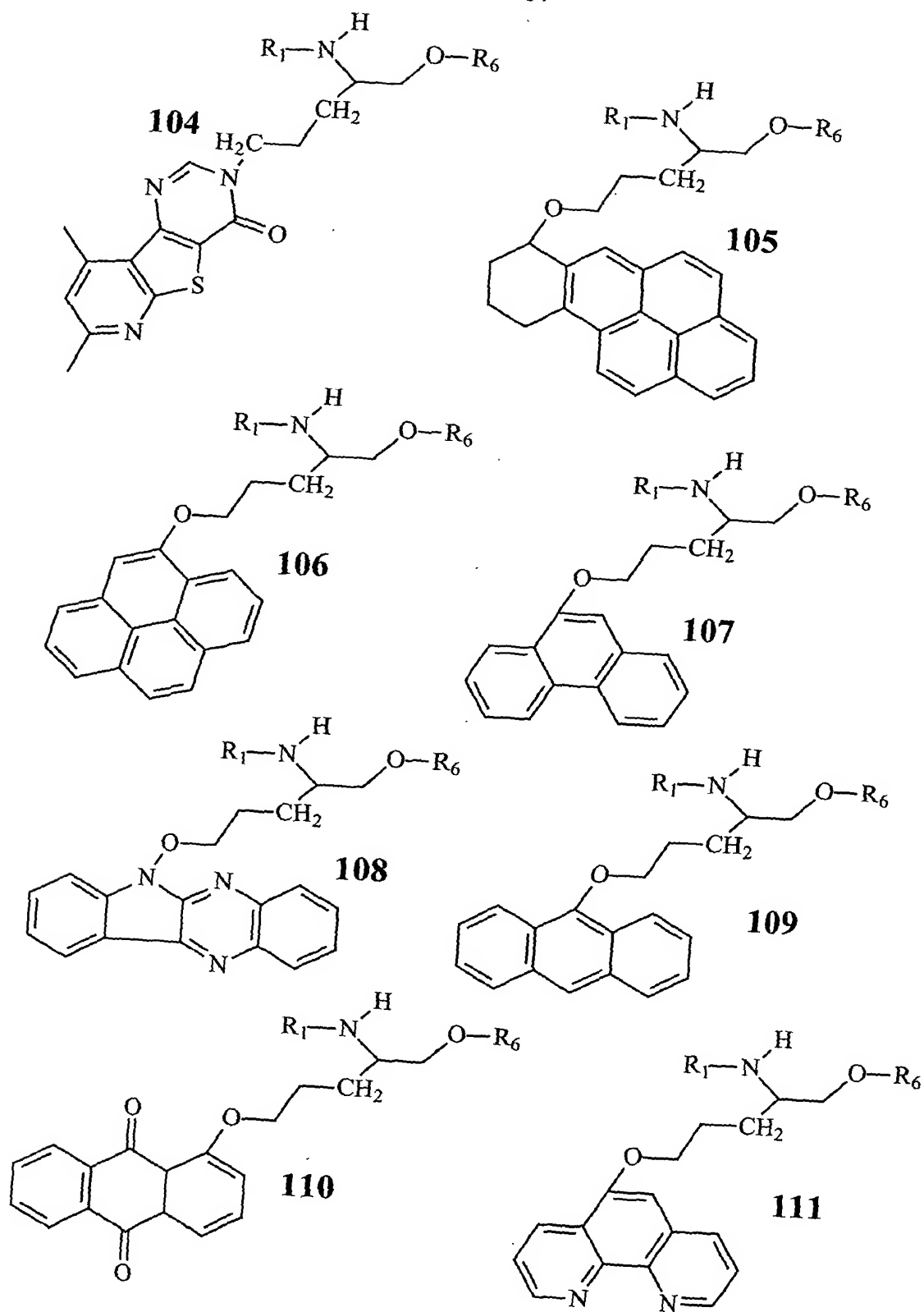
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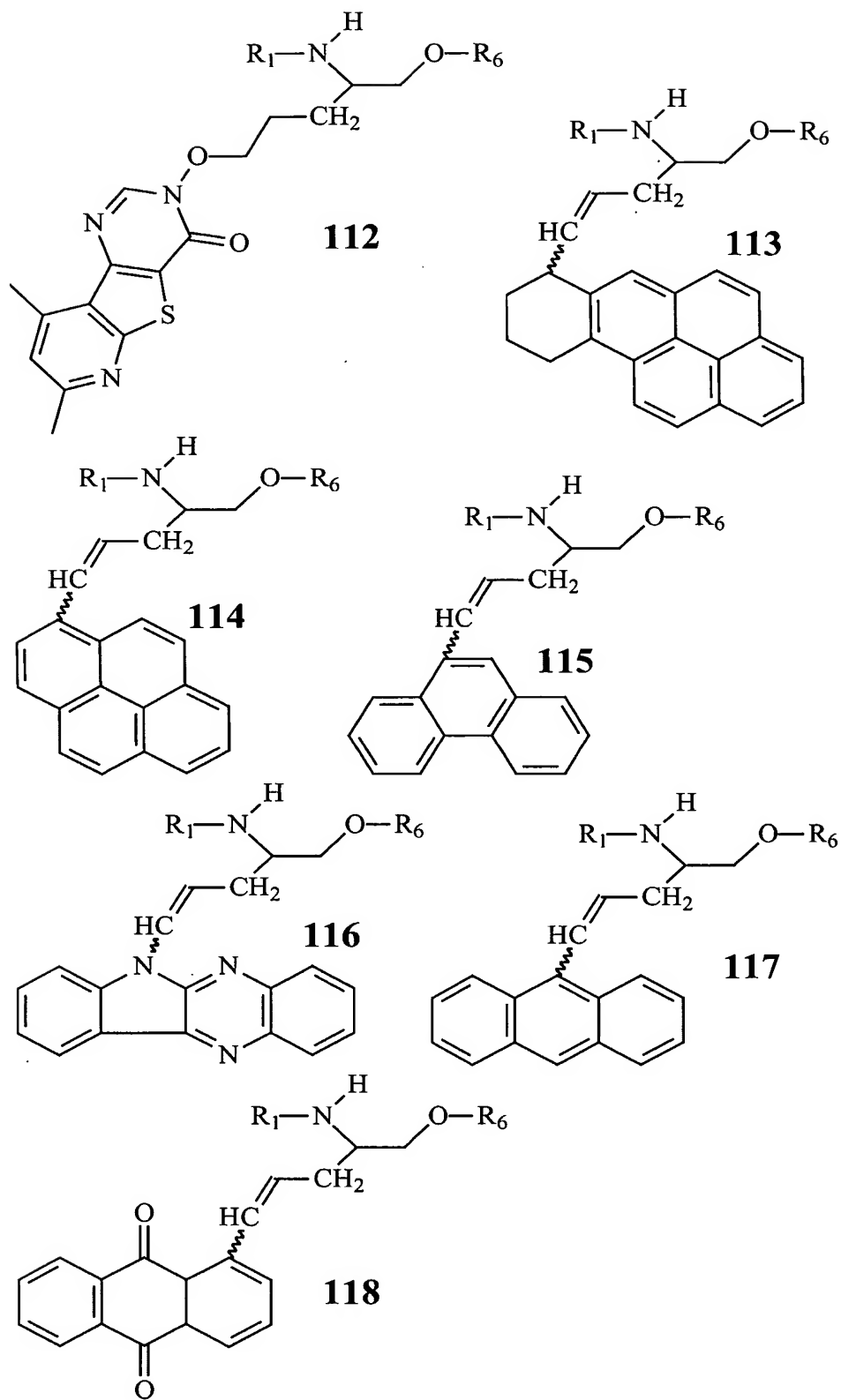
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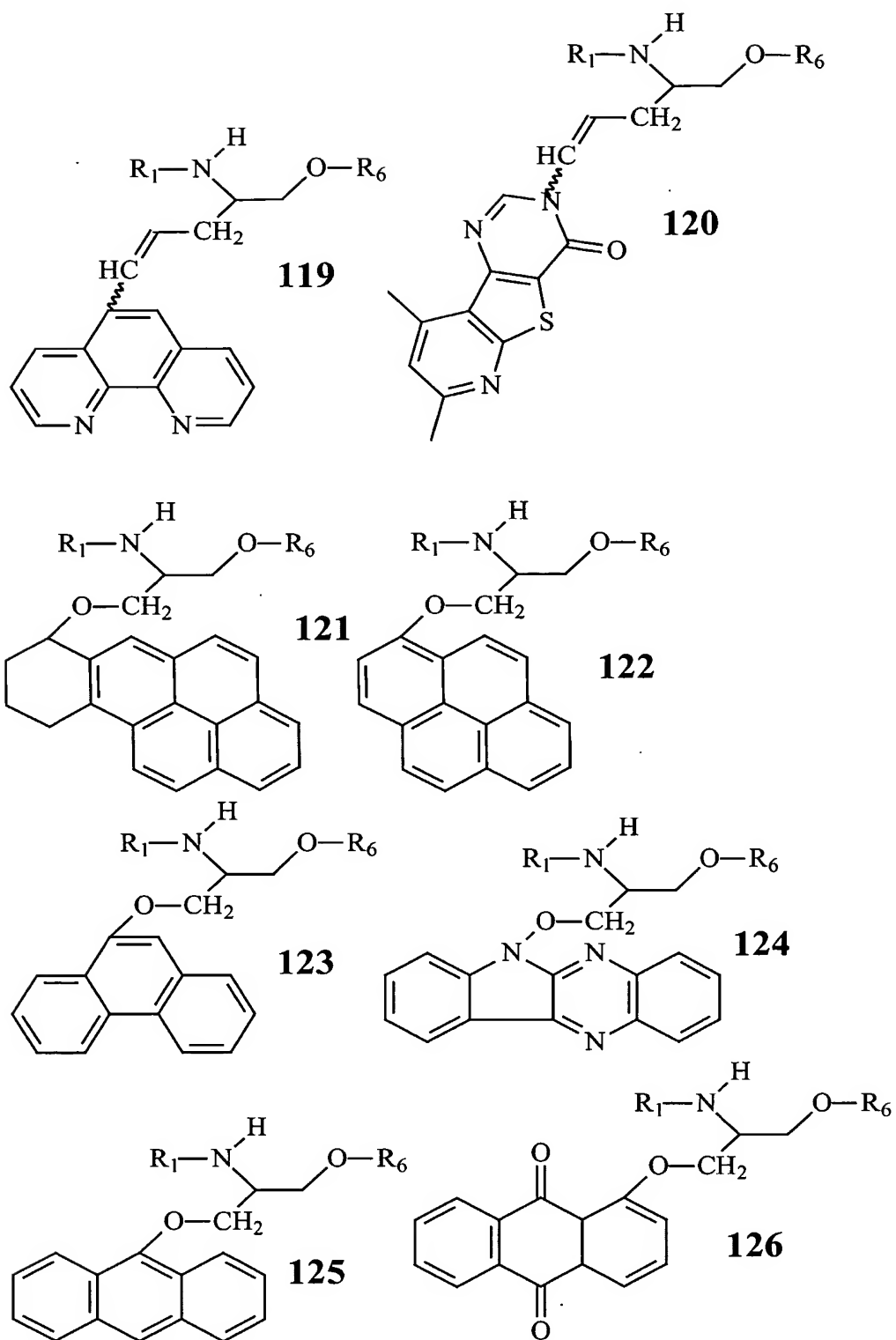
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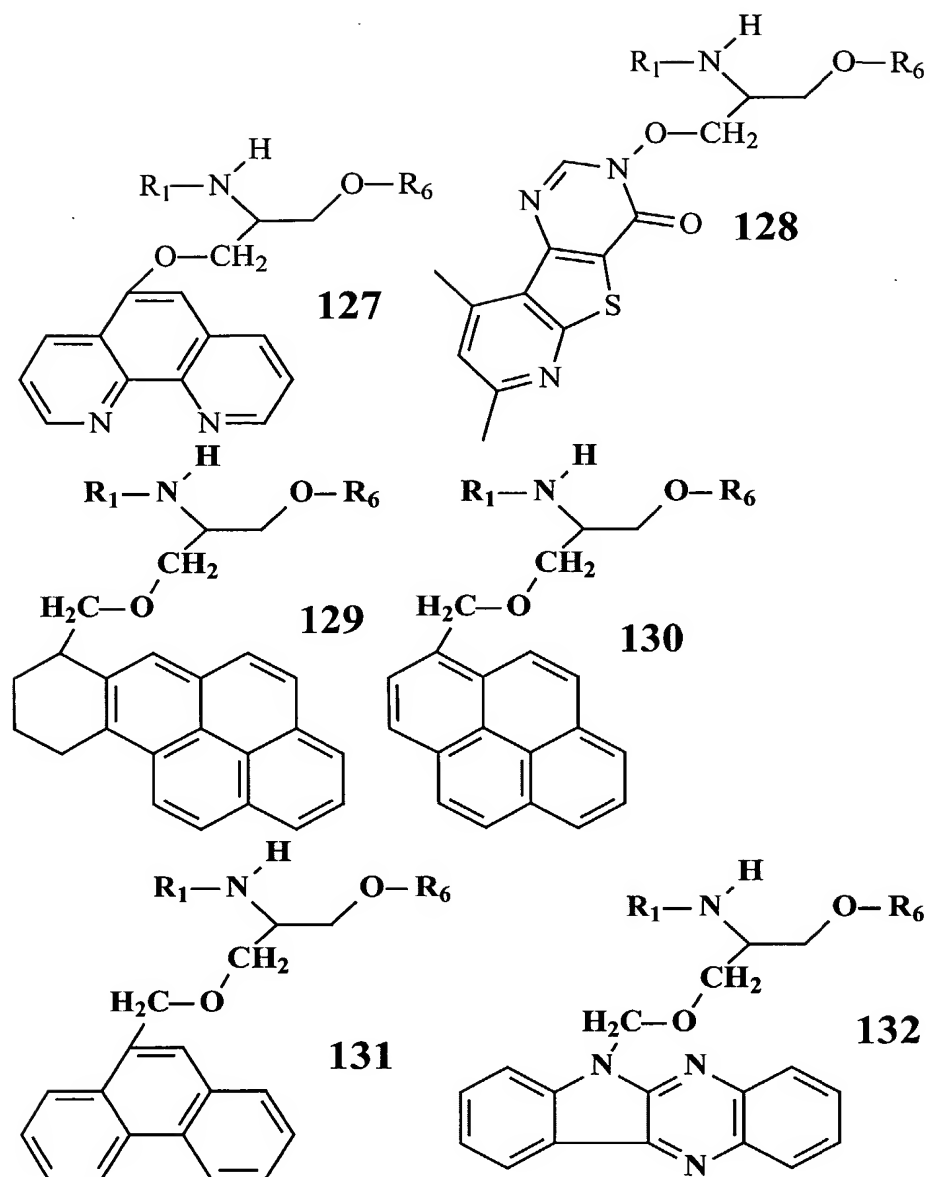
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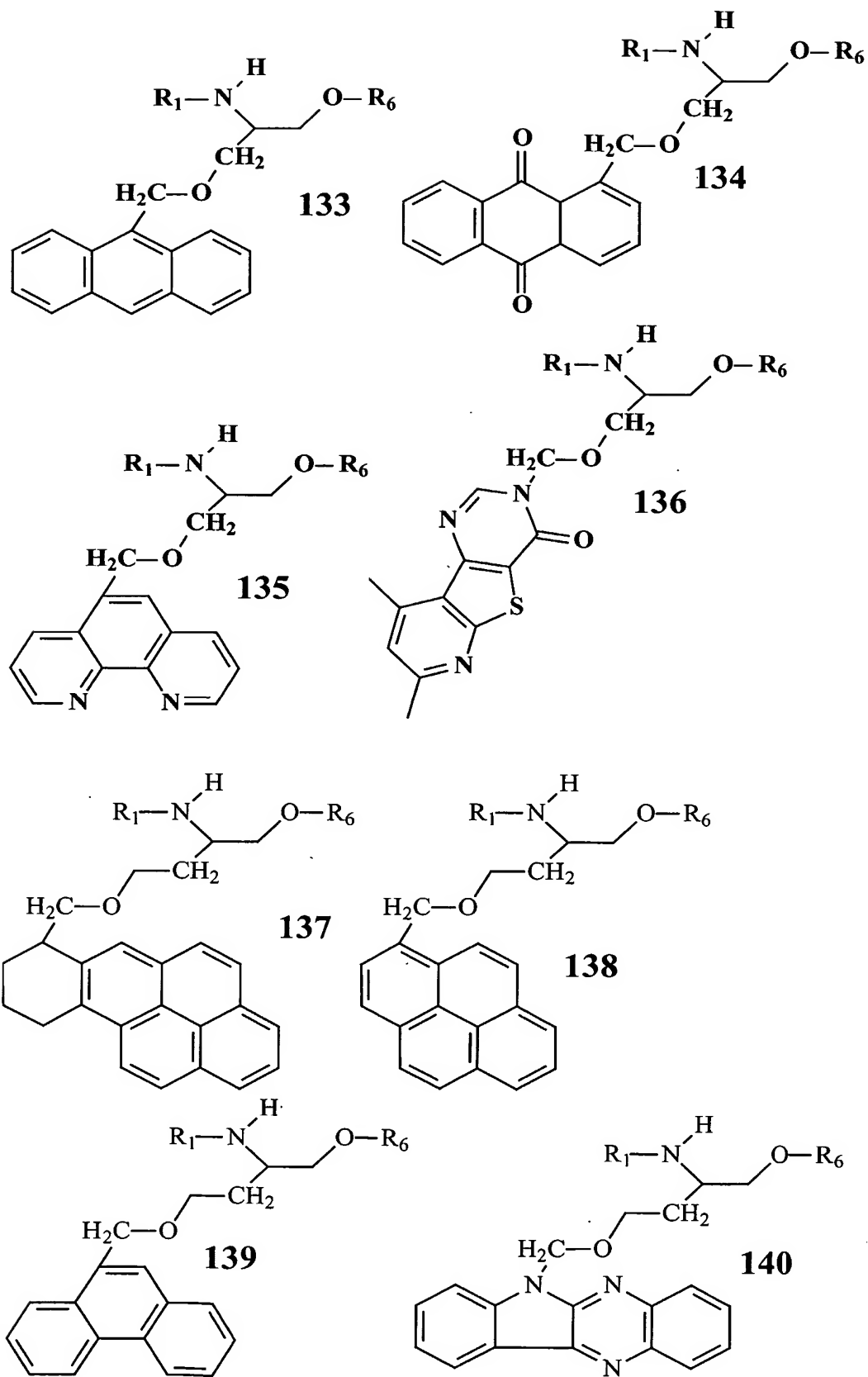


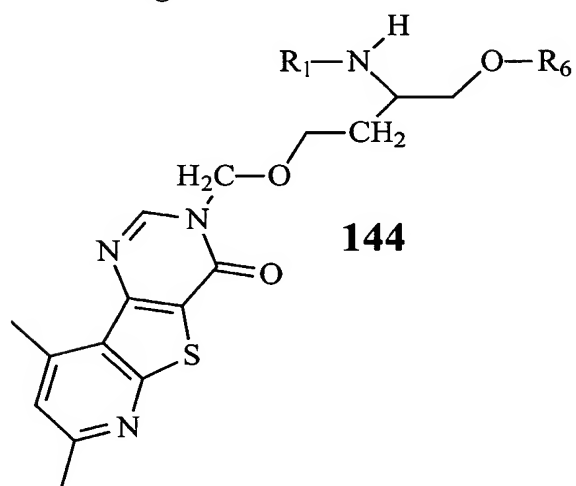
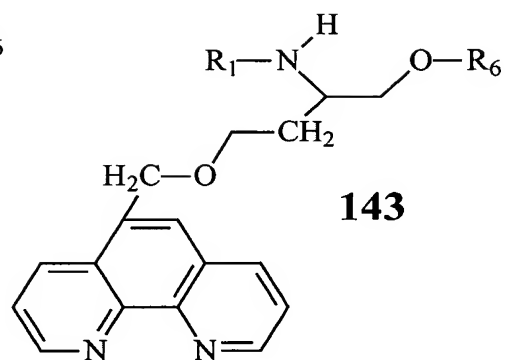
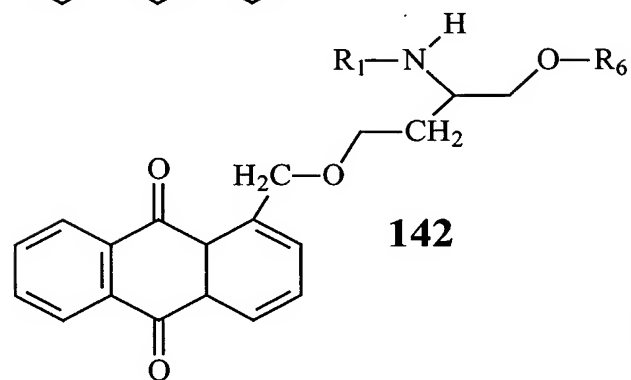
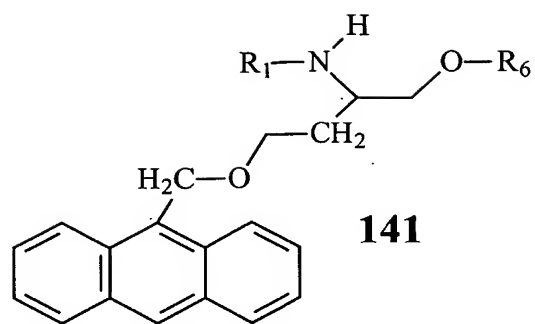
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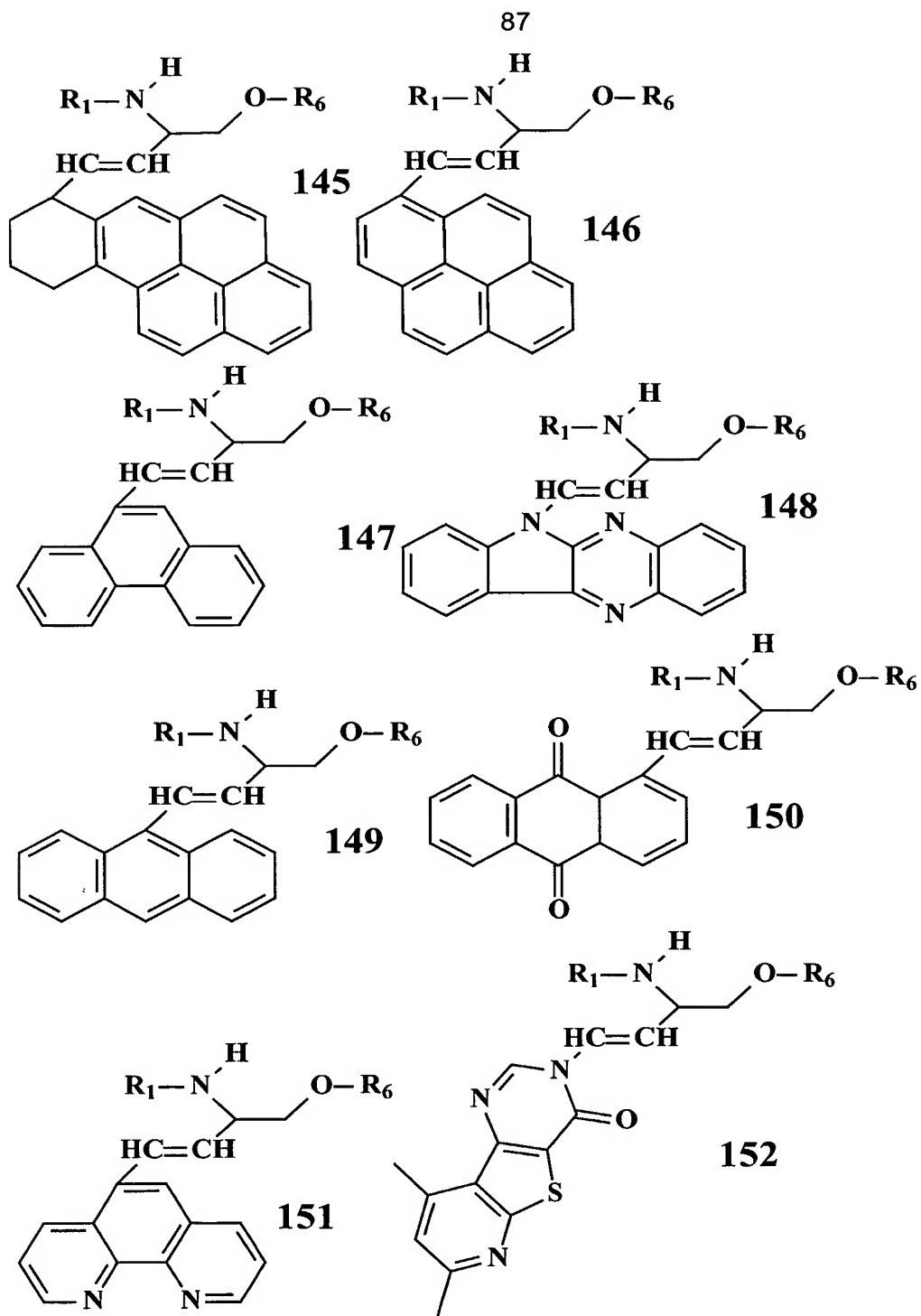


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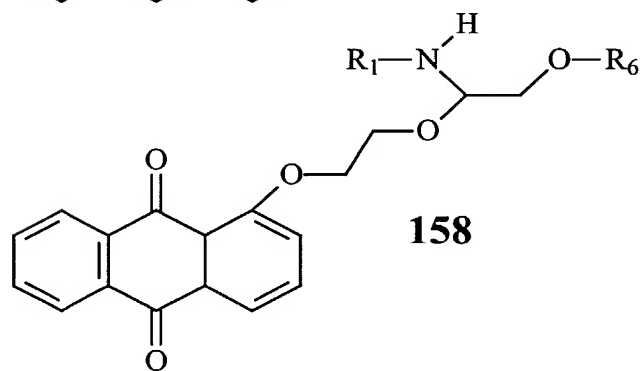
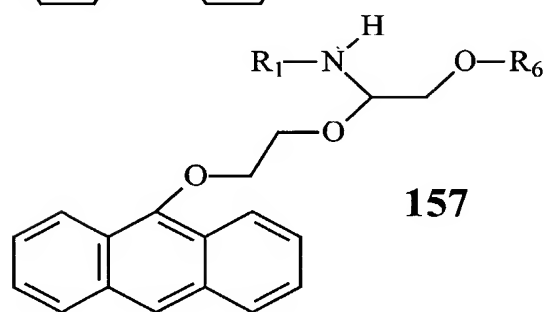
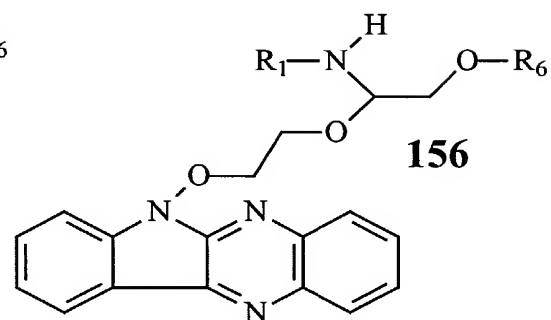
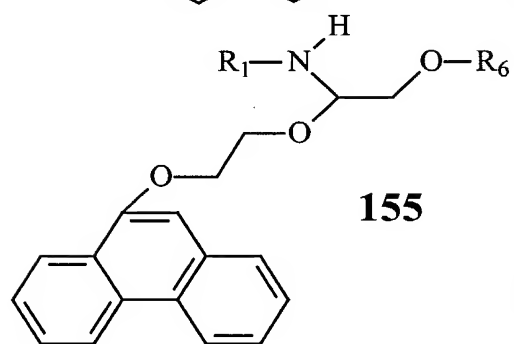
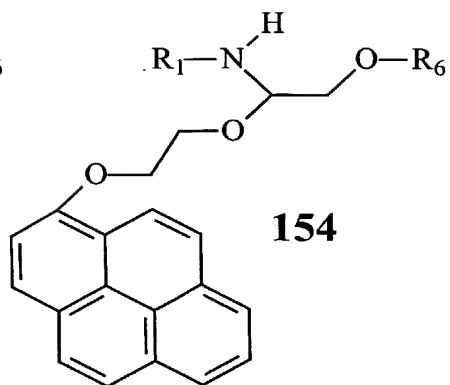
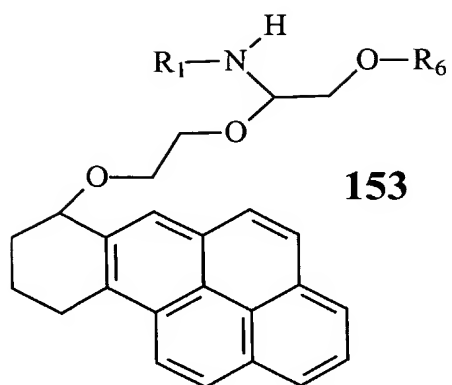


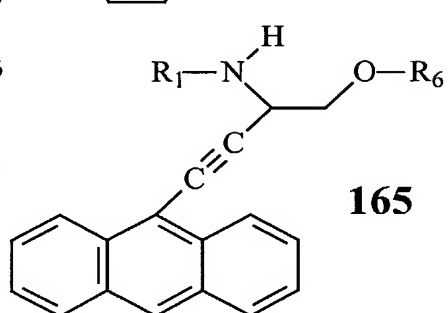
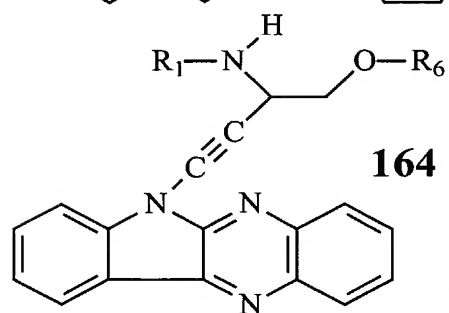
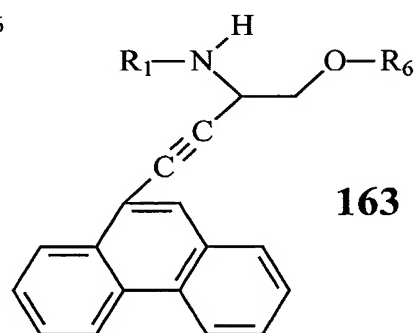
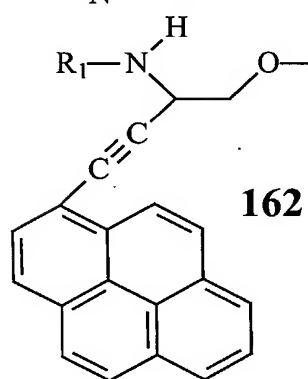
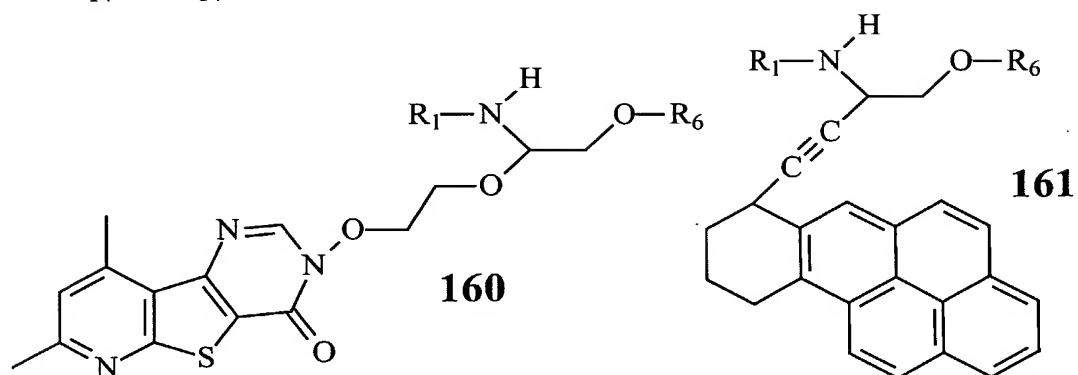
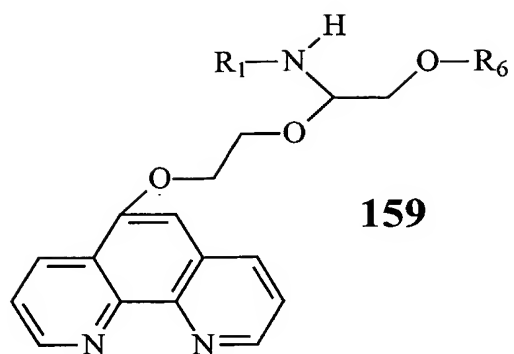




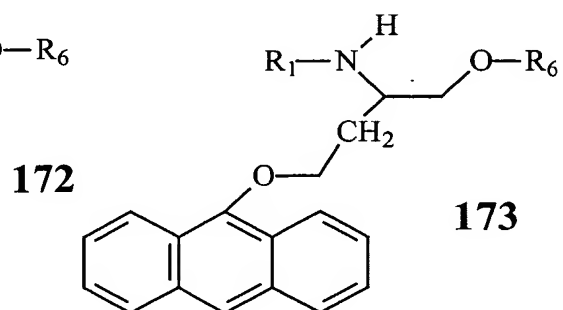
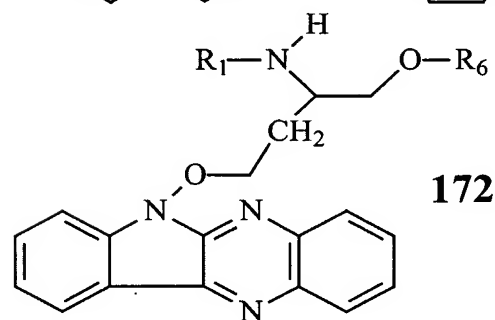
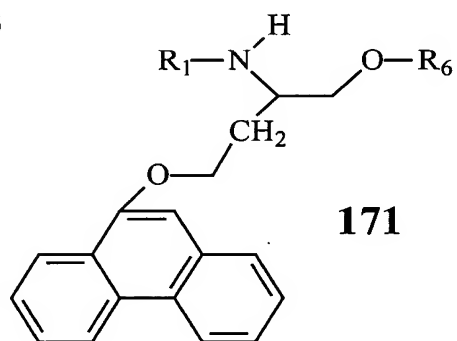
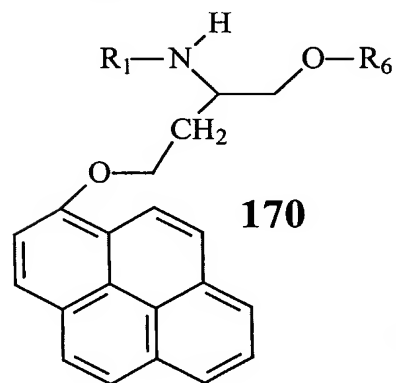
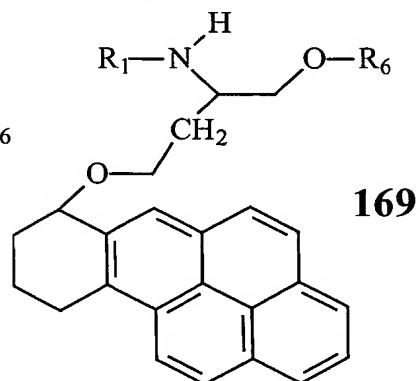
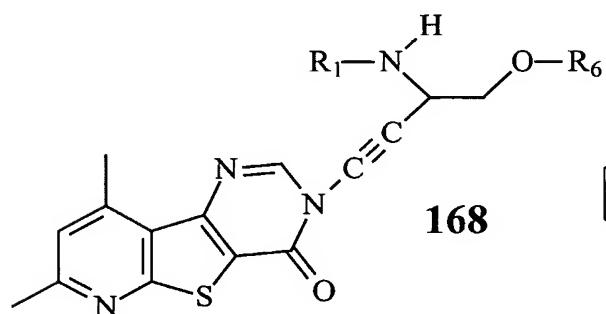
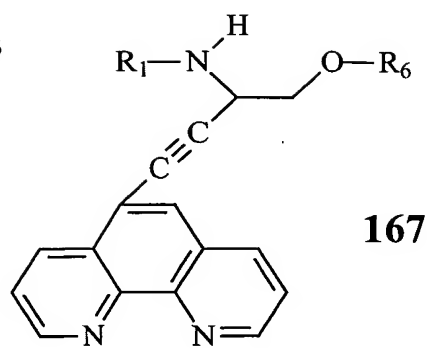
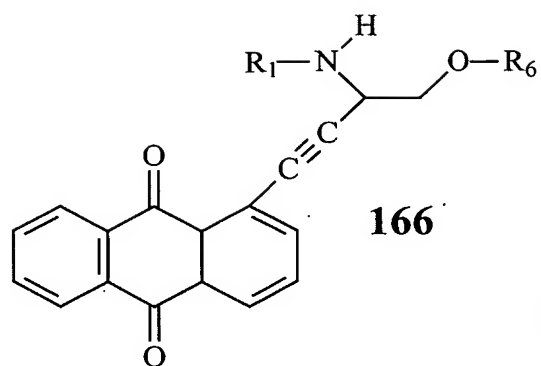


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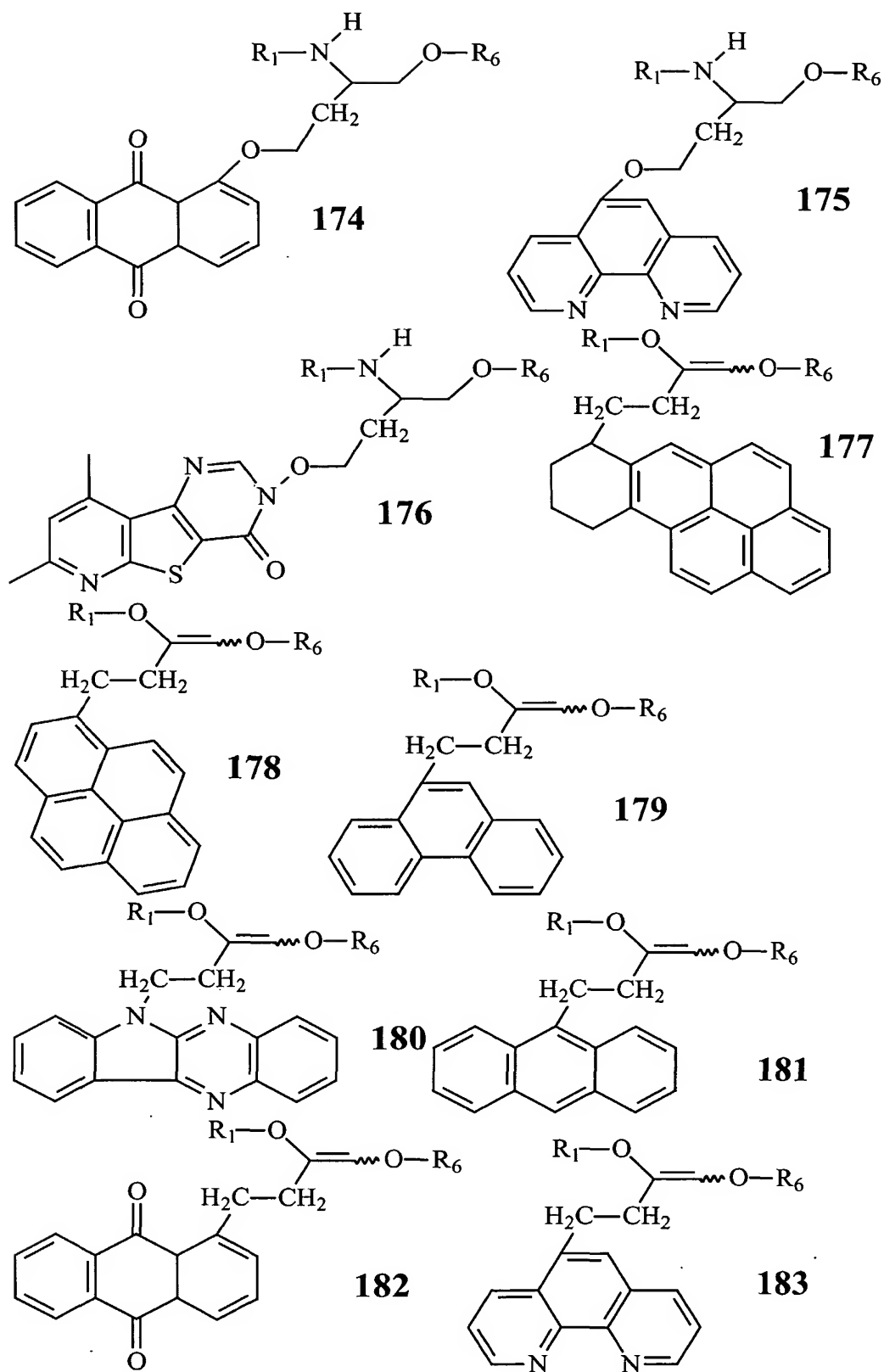




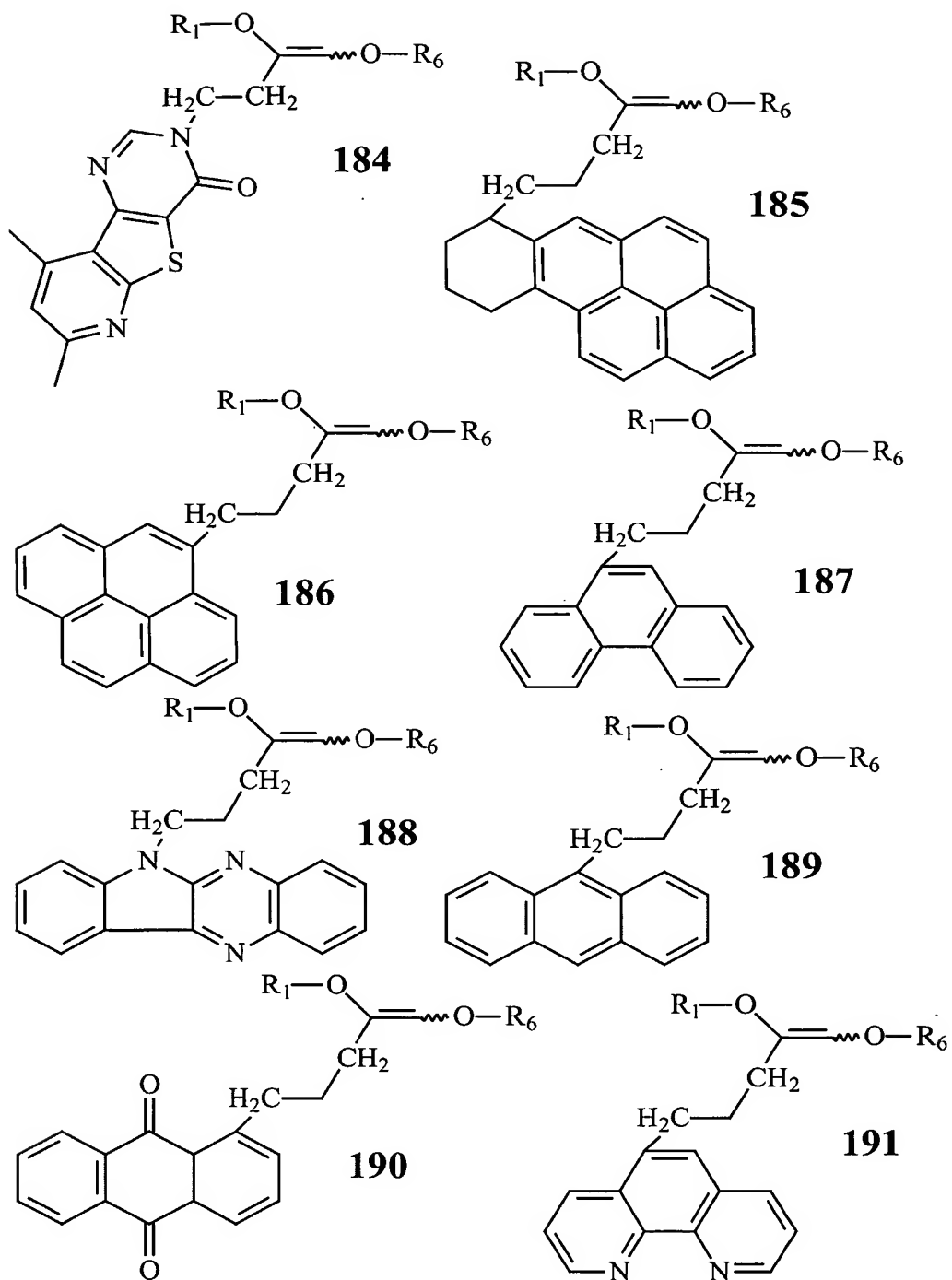
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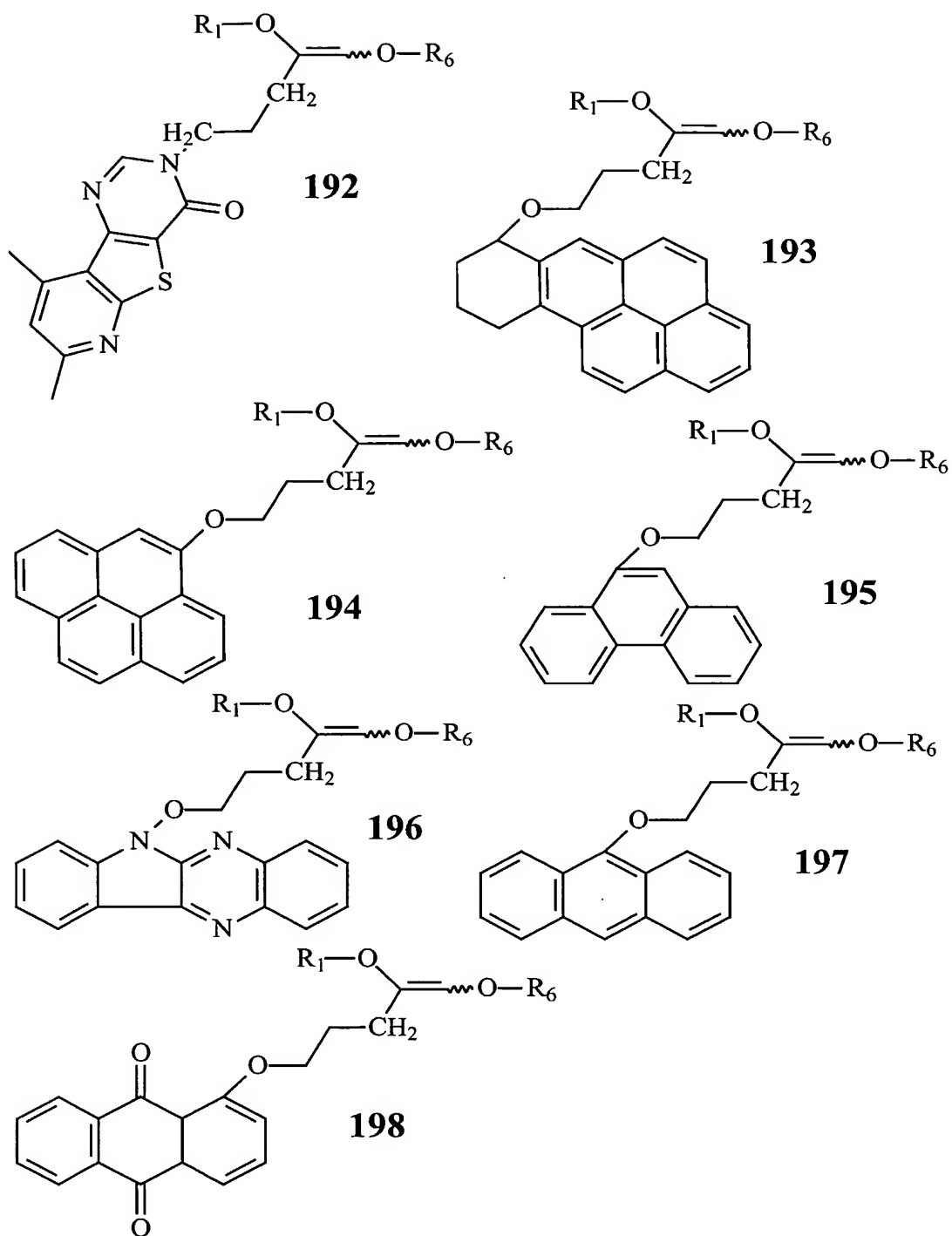


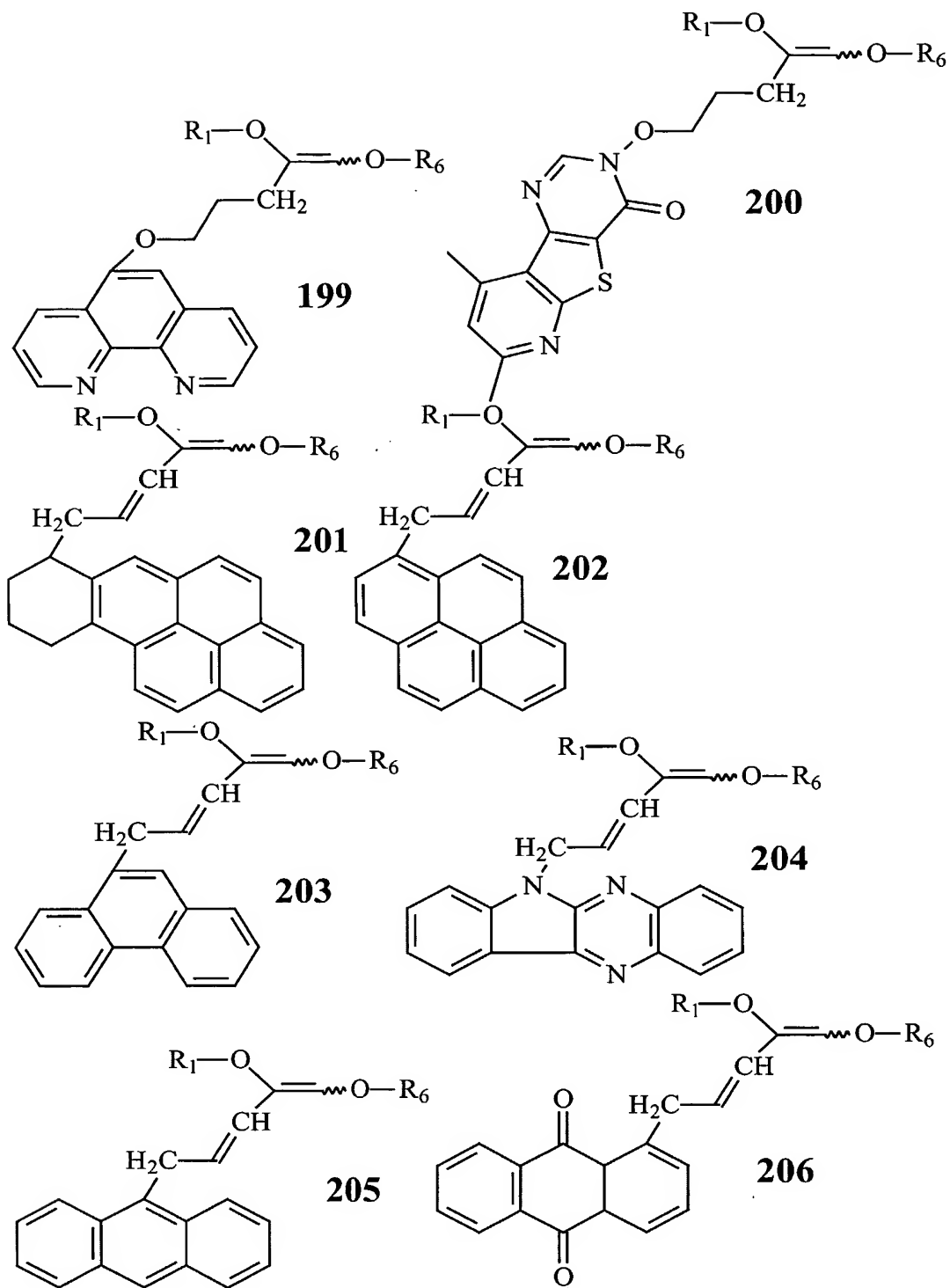
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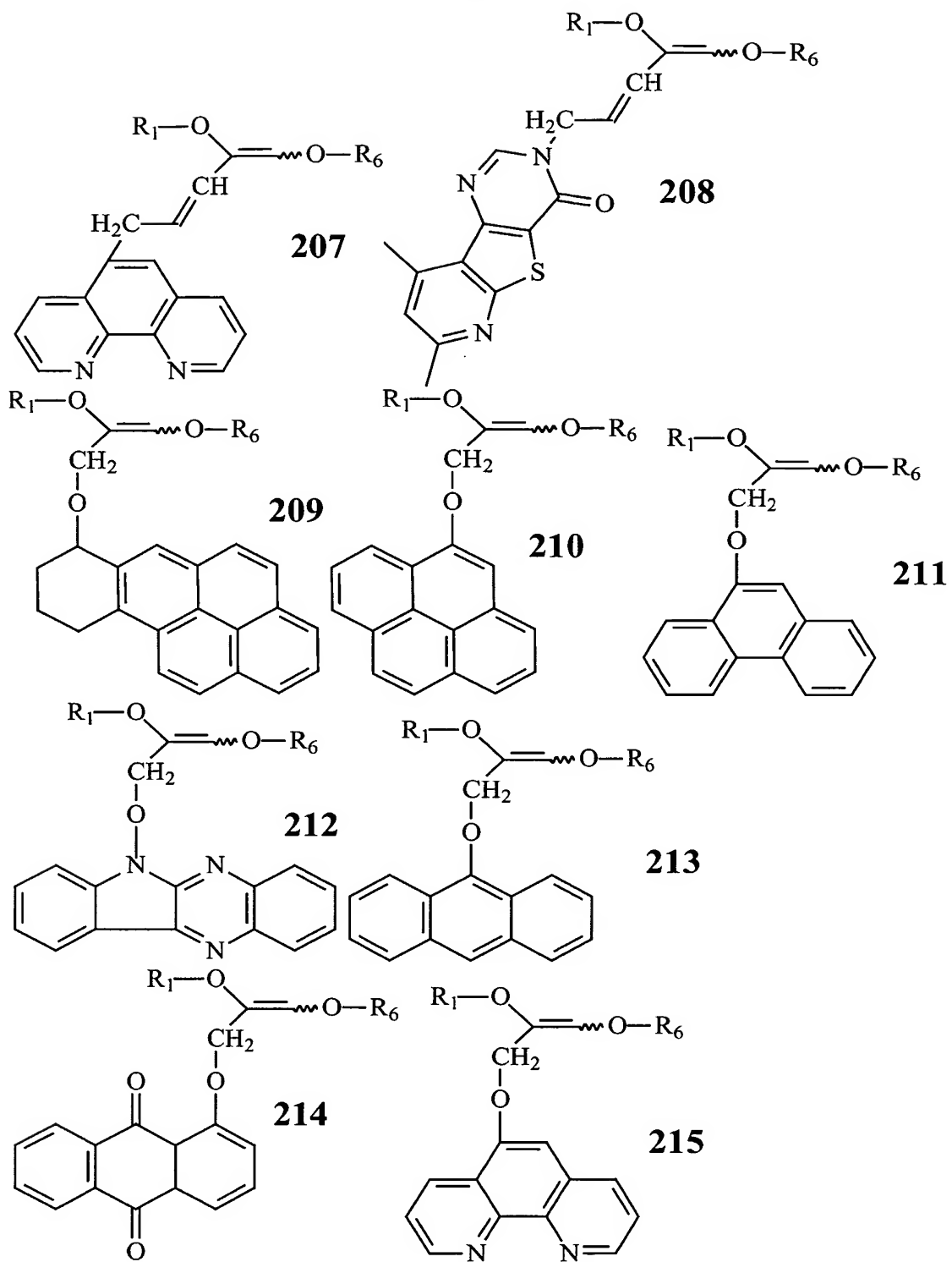
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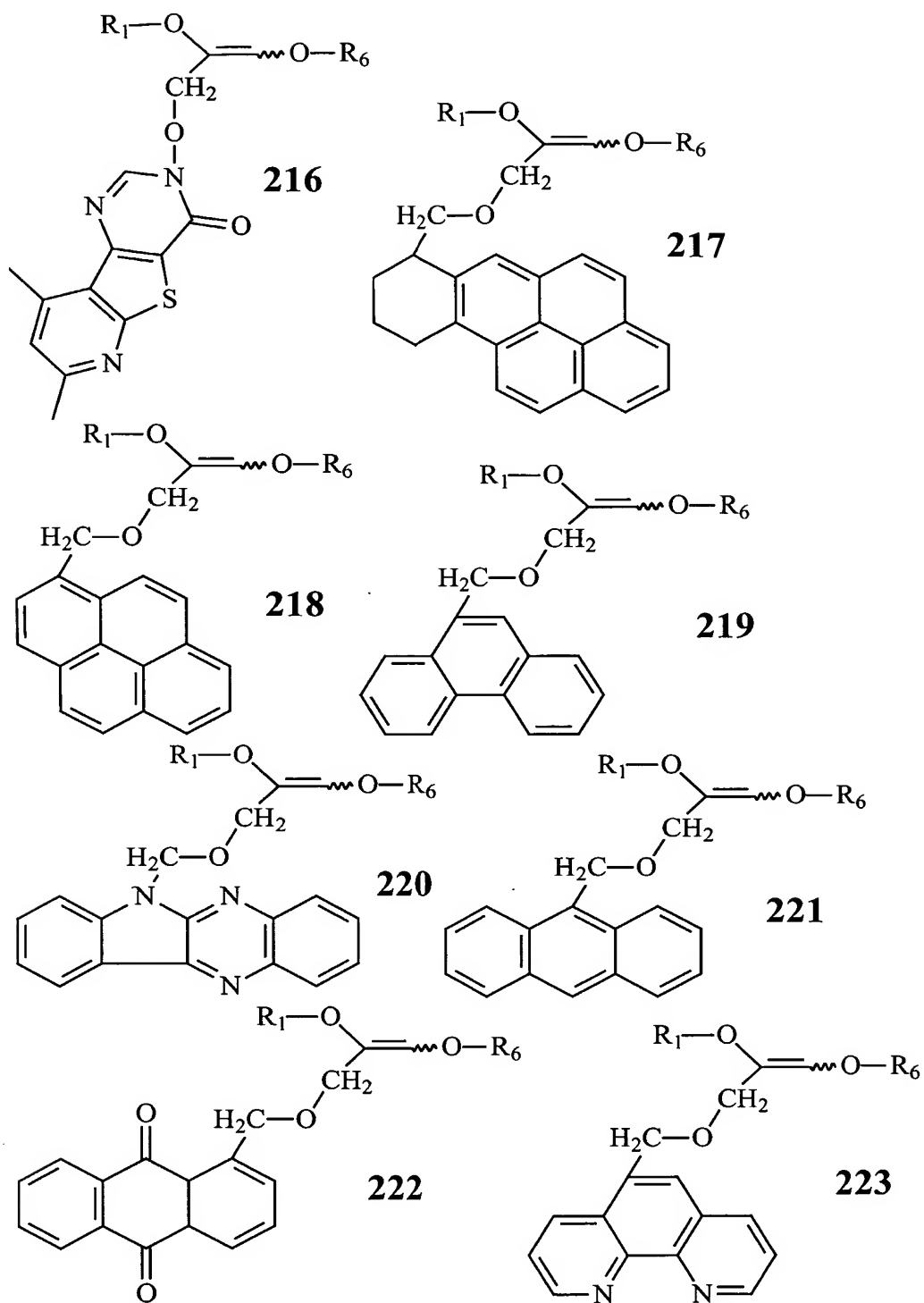


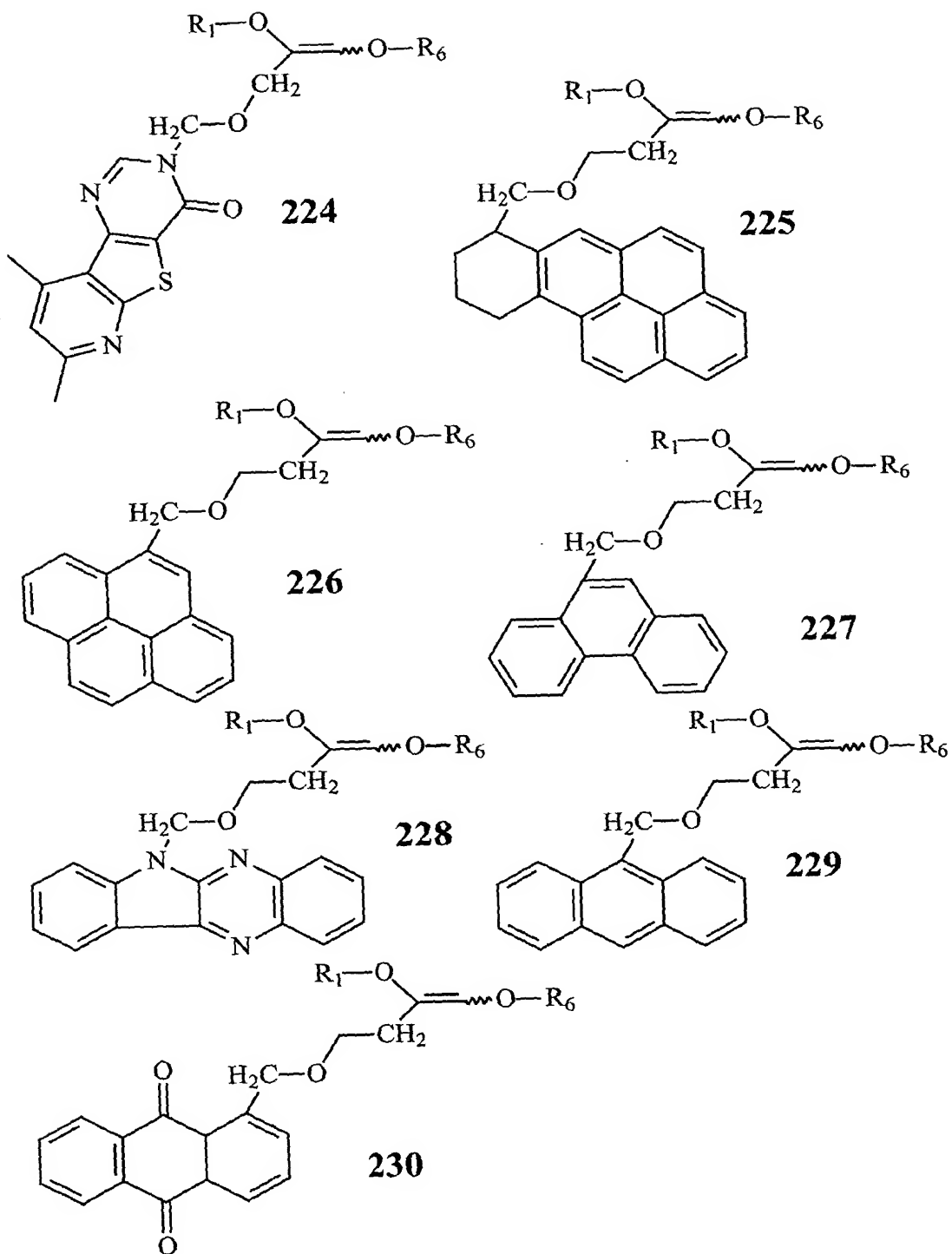


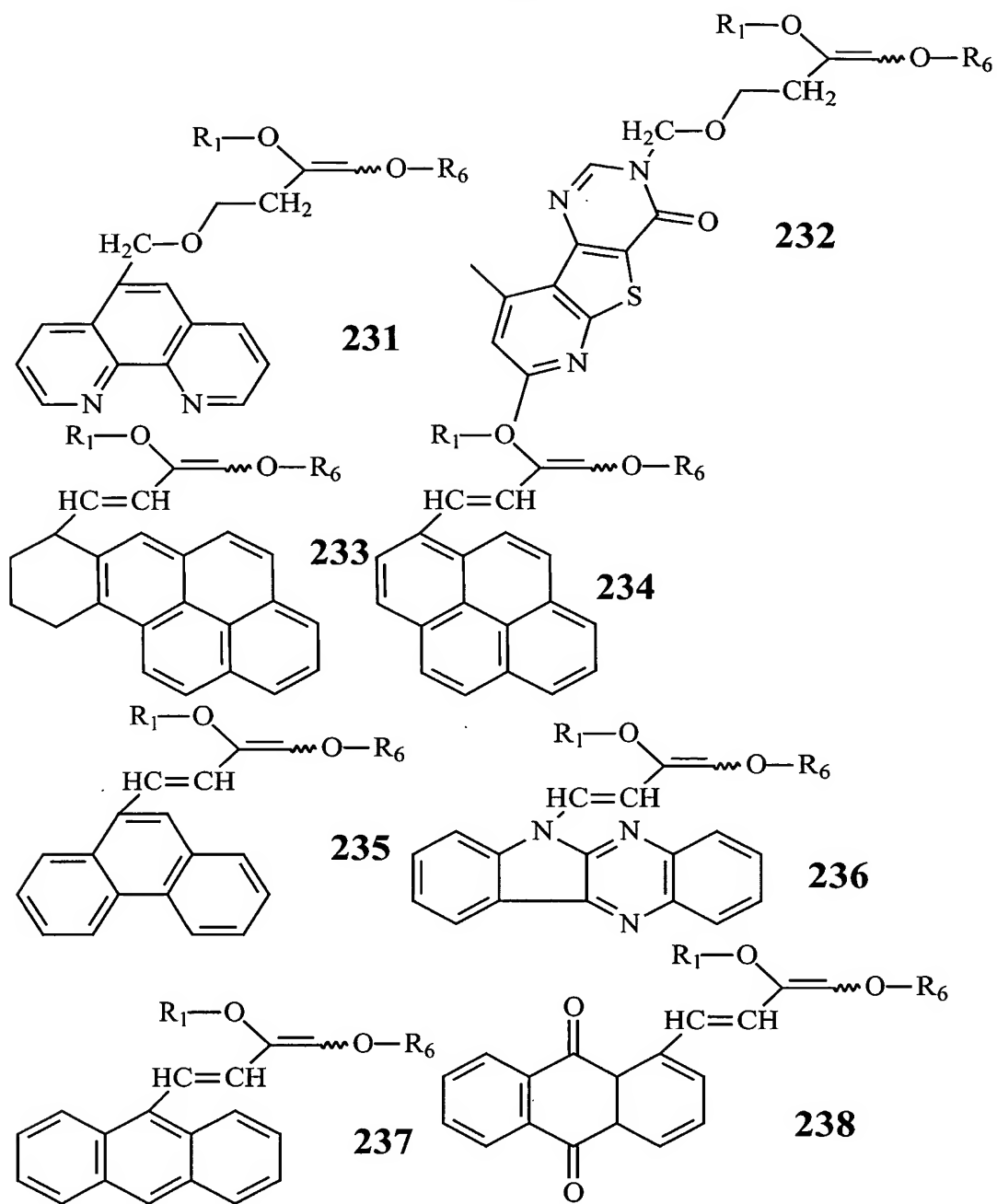


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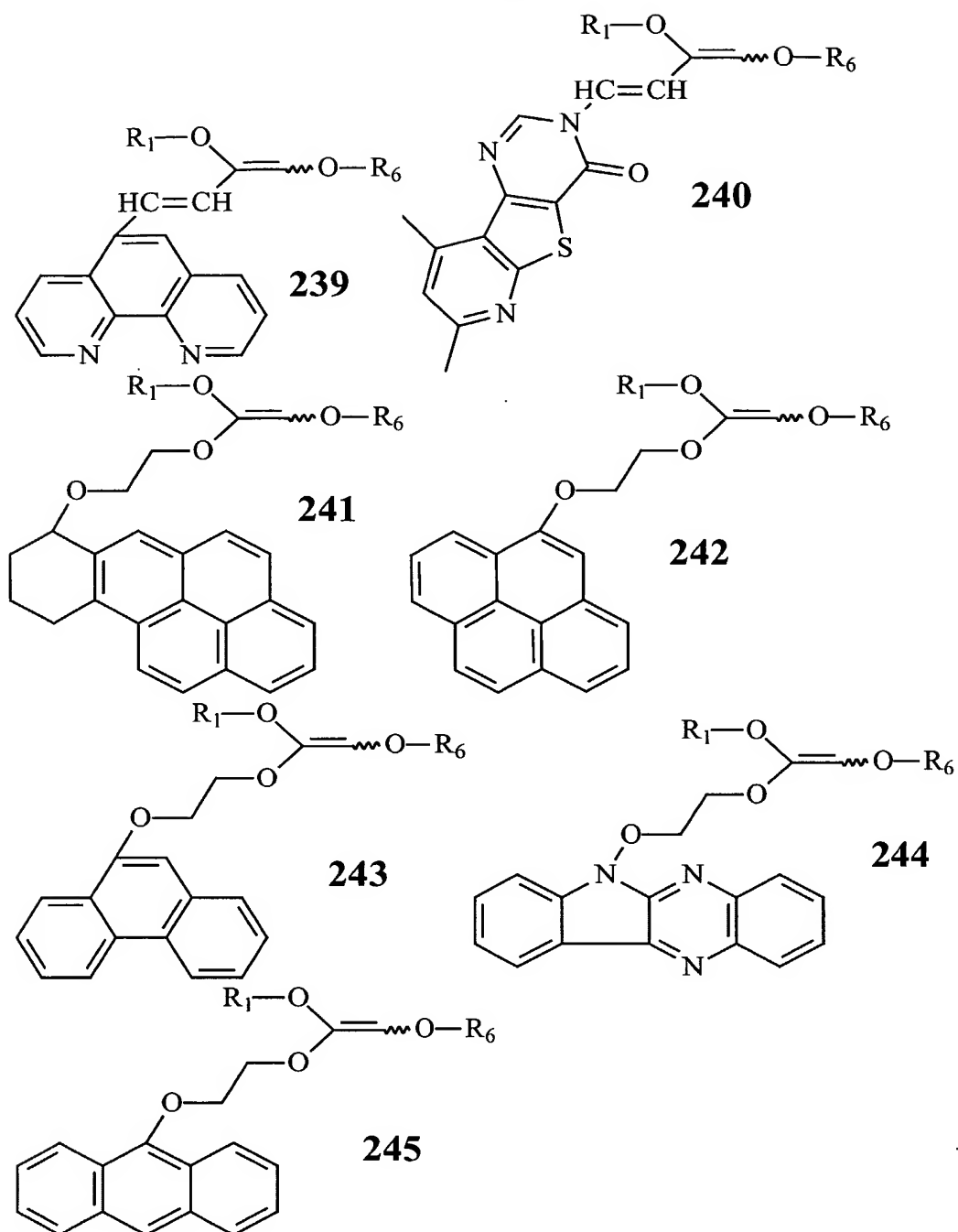




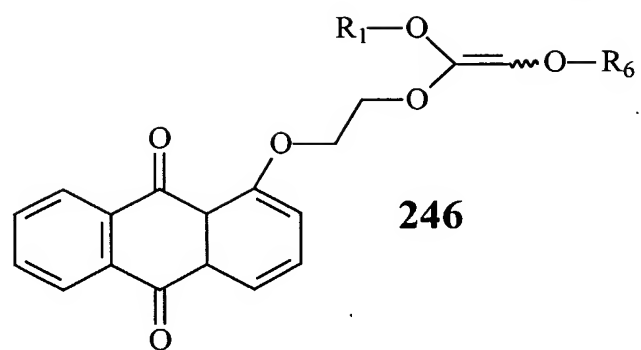




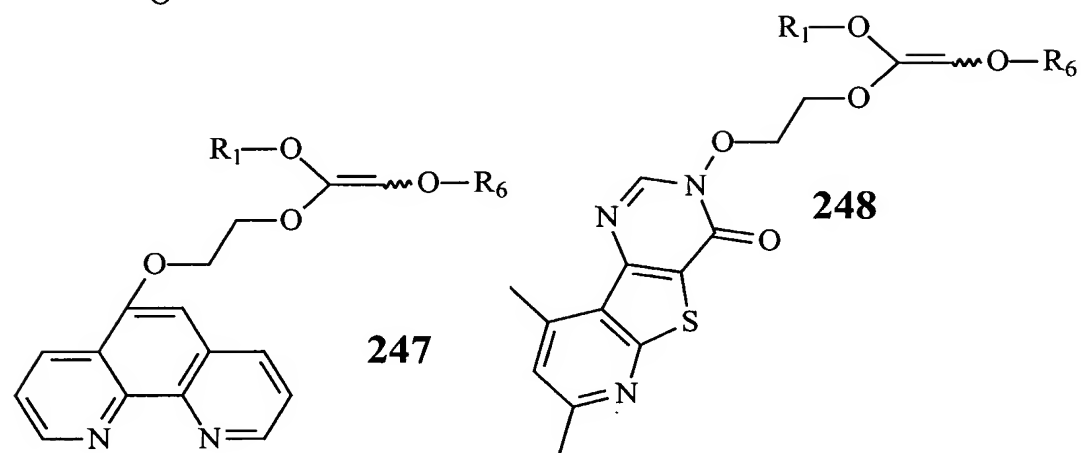
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100



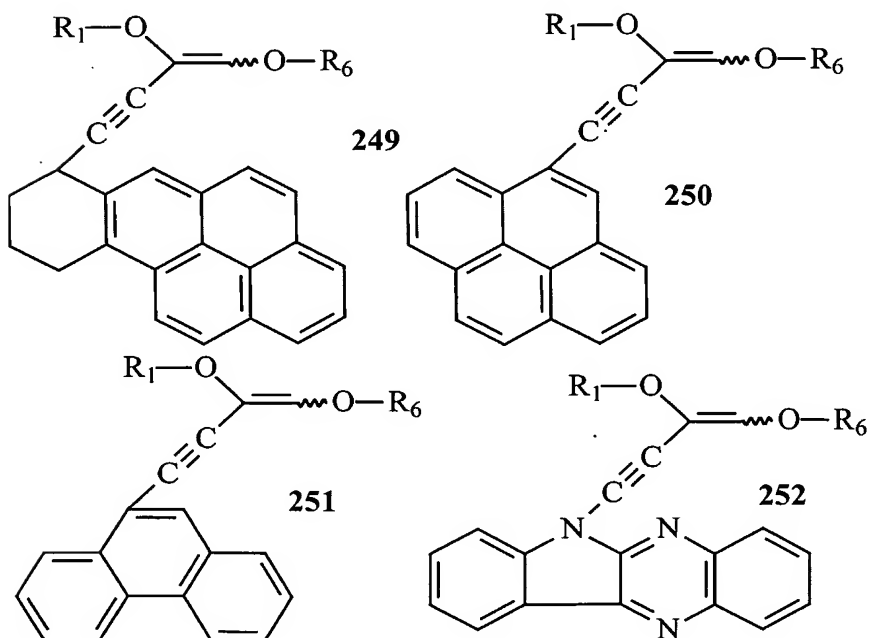
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247

248

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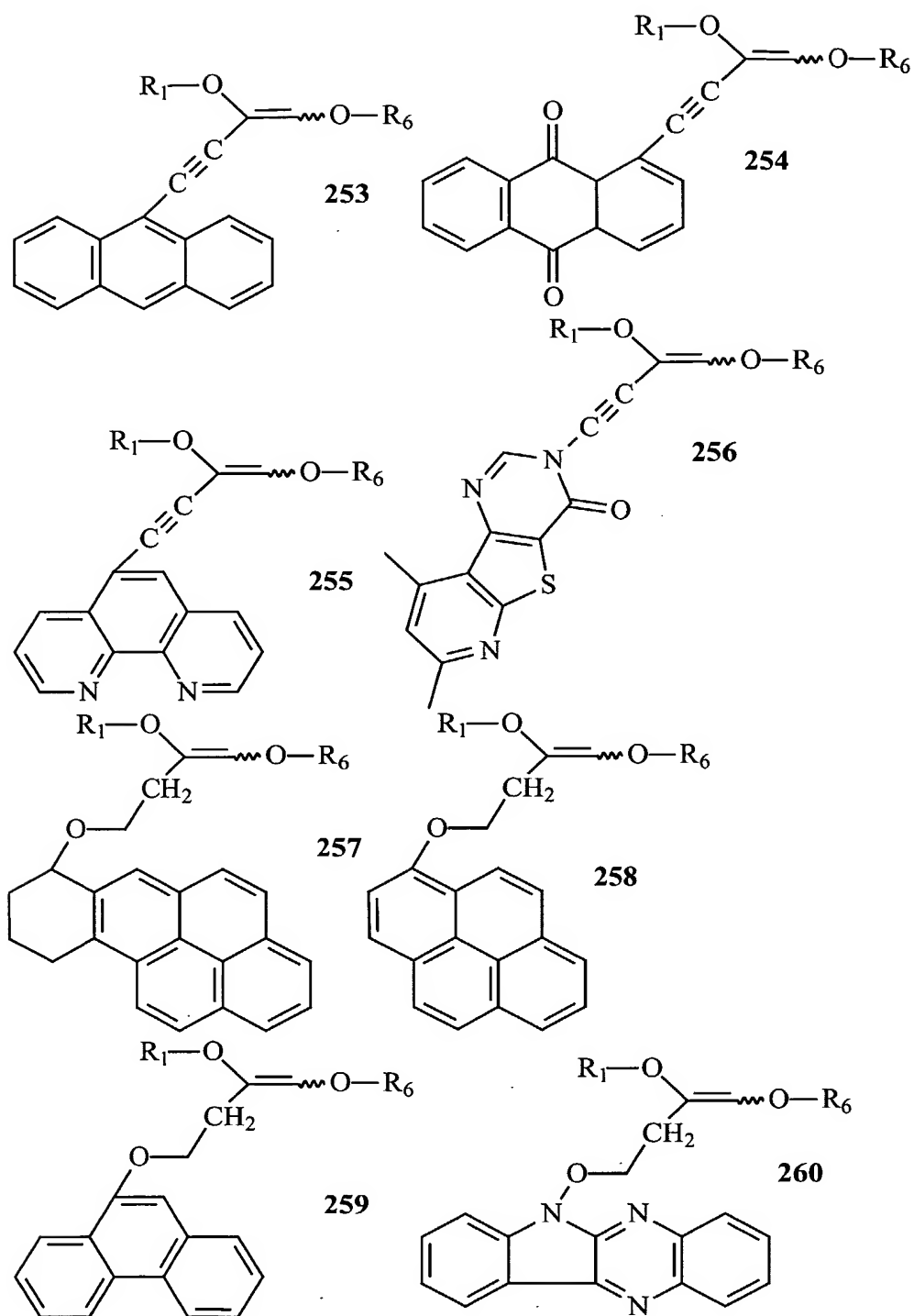
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250

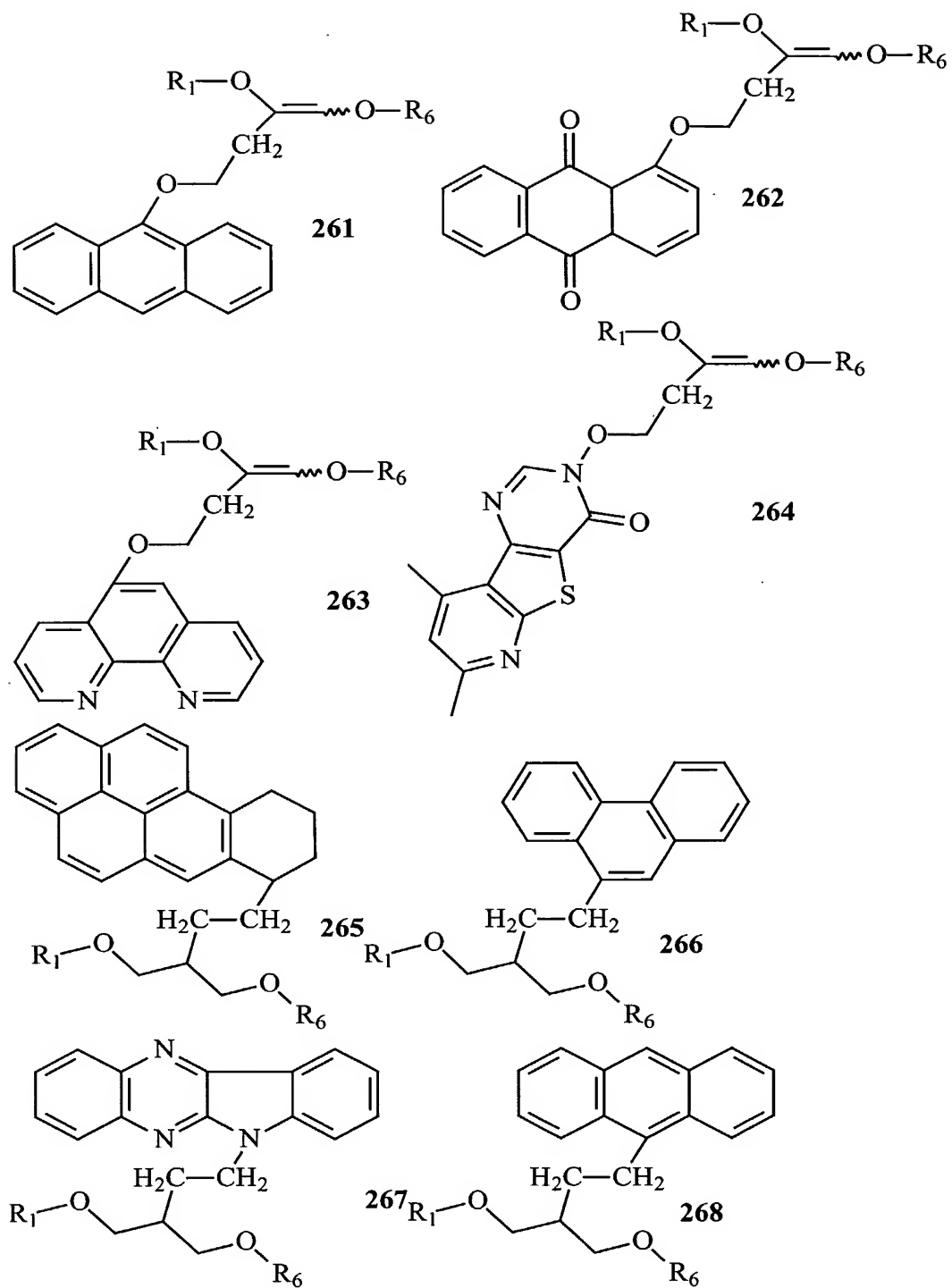
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252

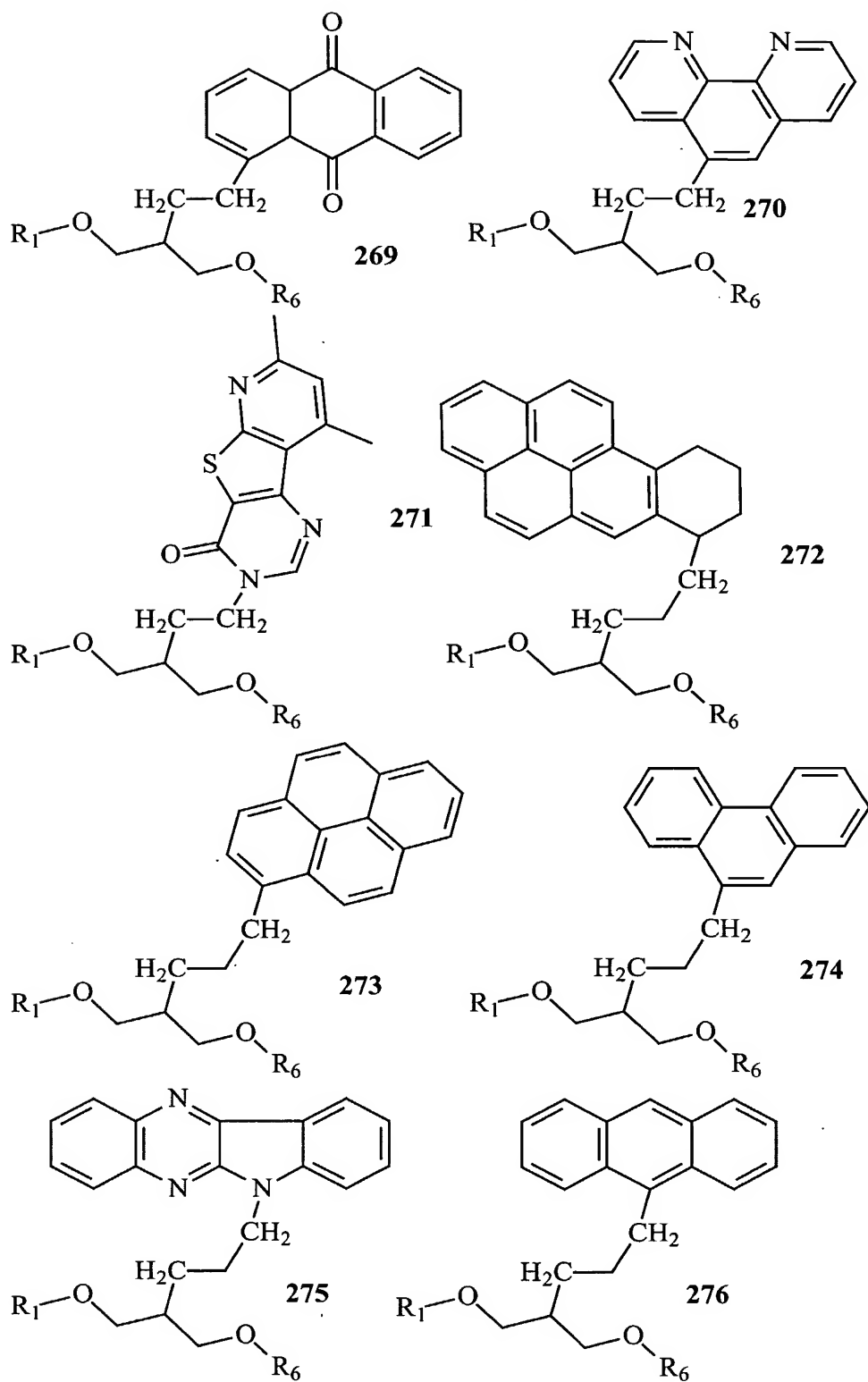
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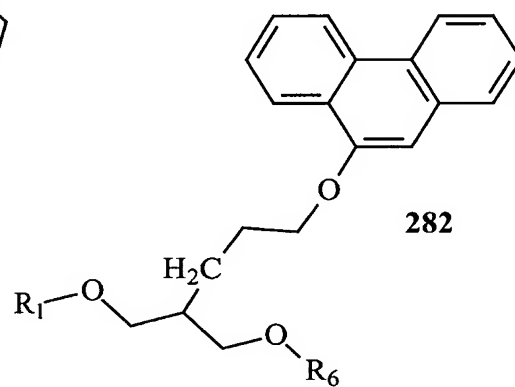
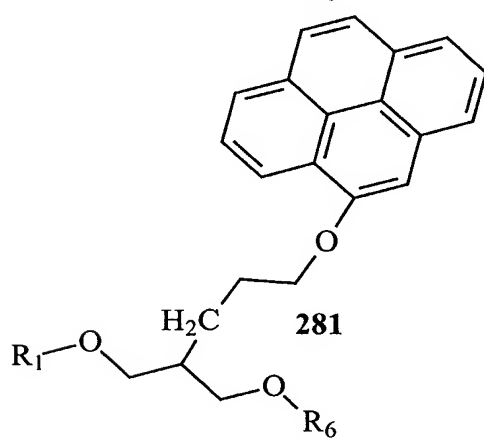
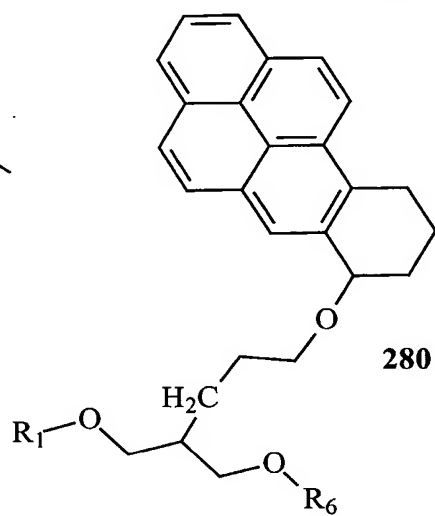
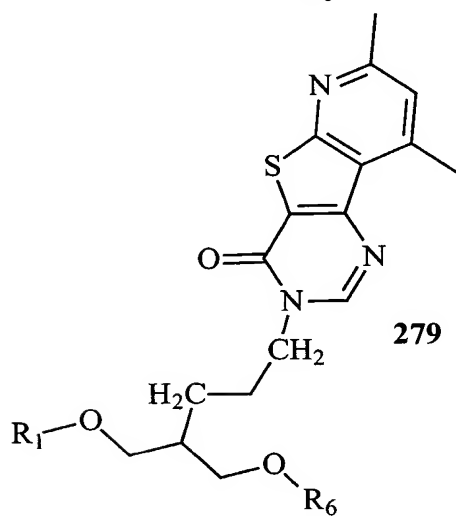
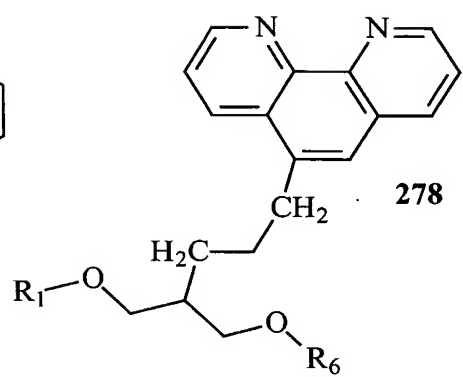
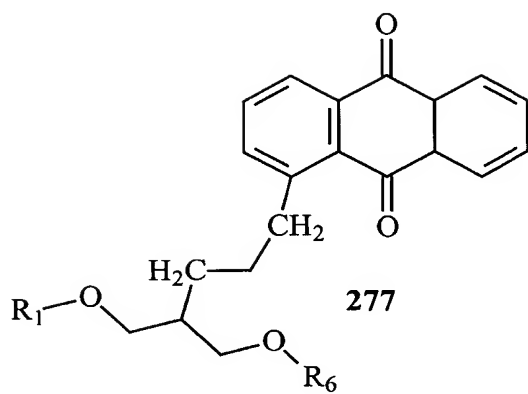
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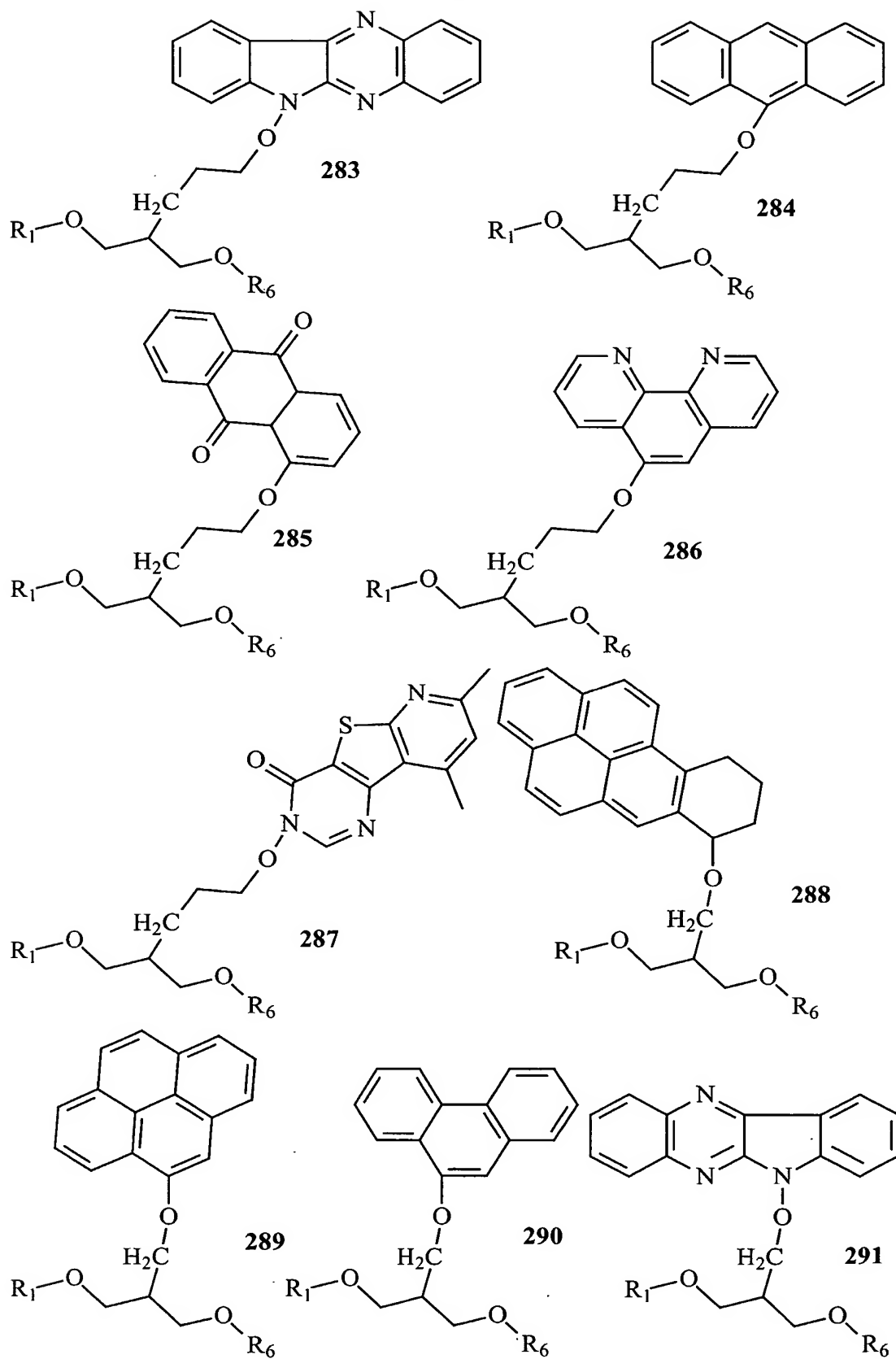
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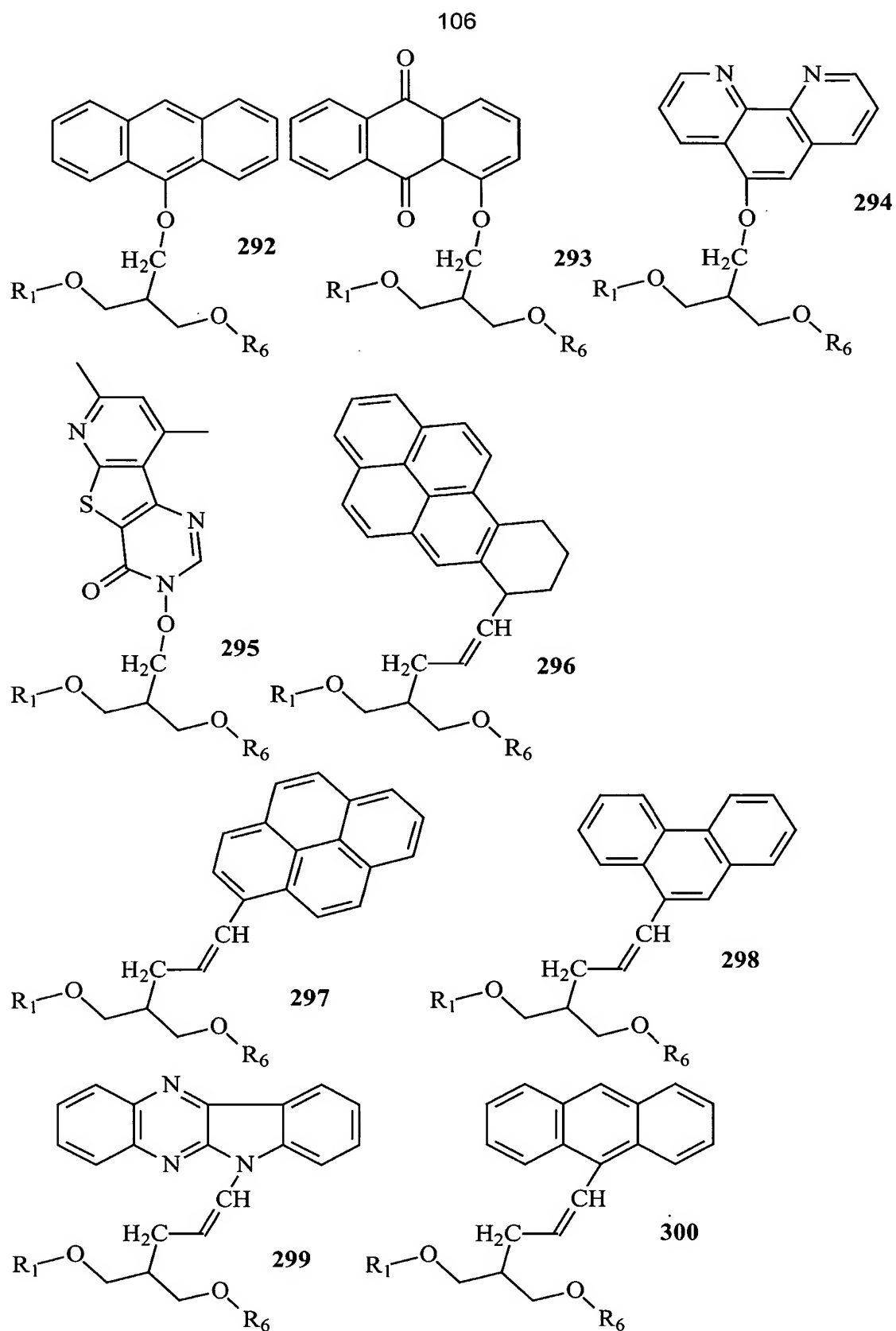


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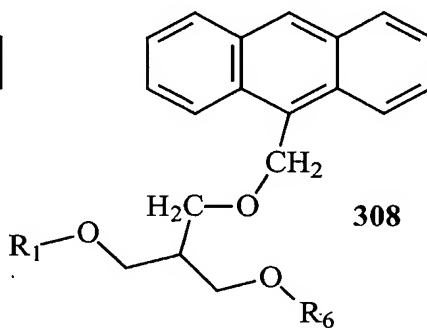
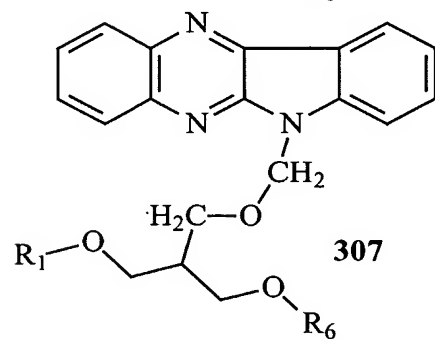
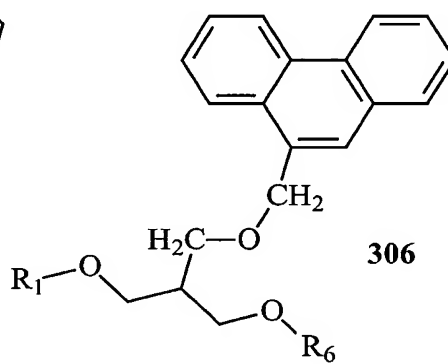
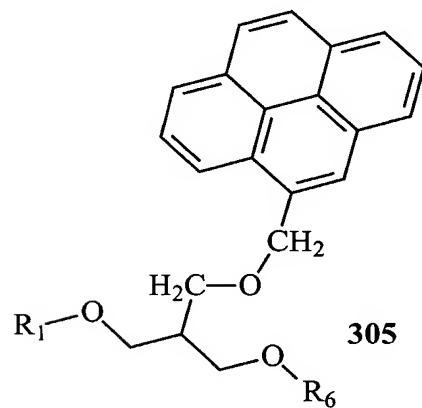
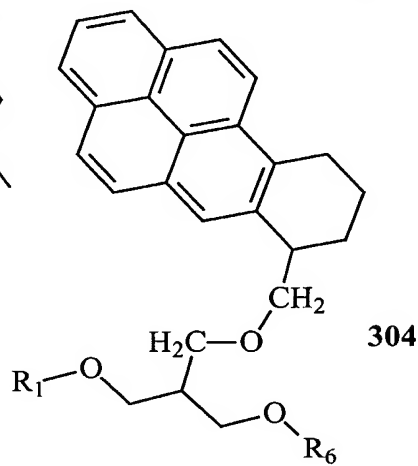
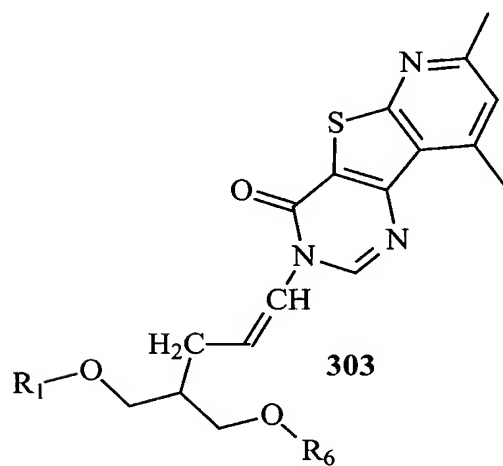
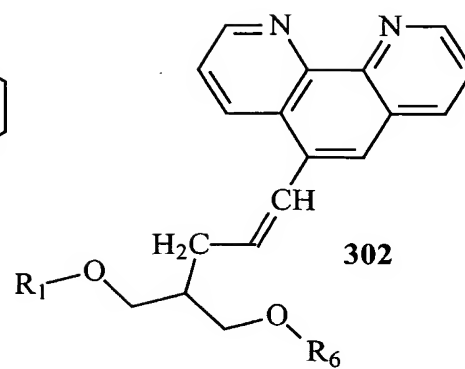
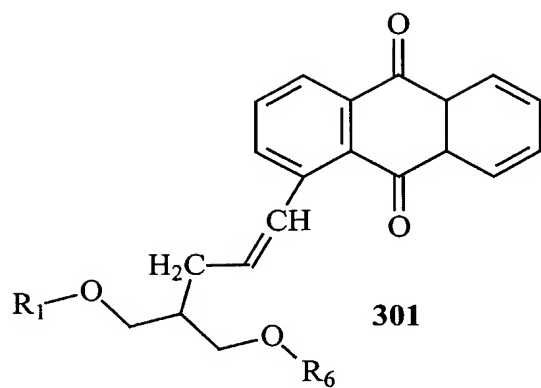


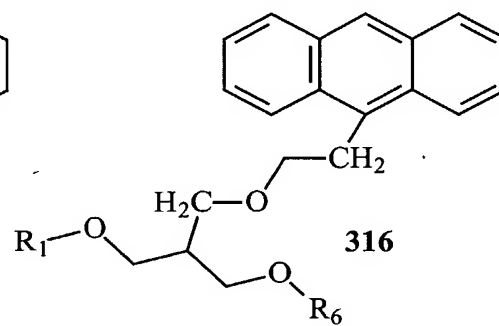
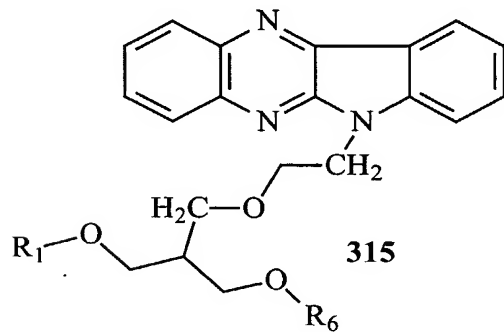
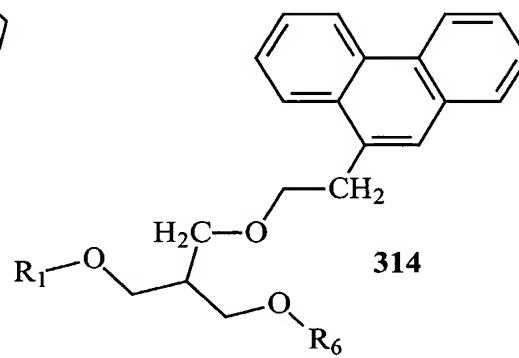
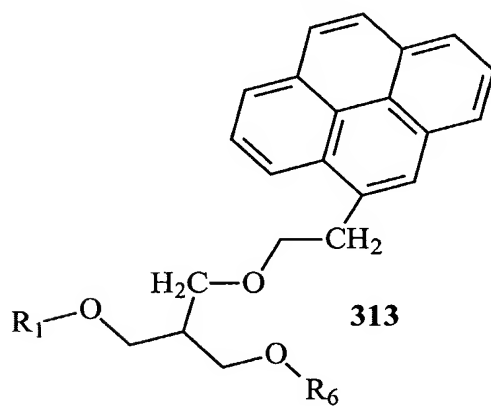
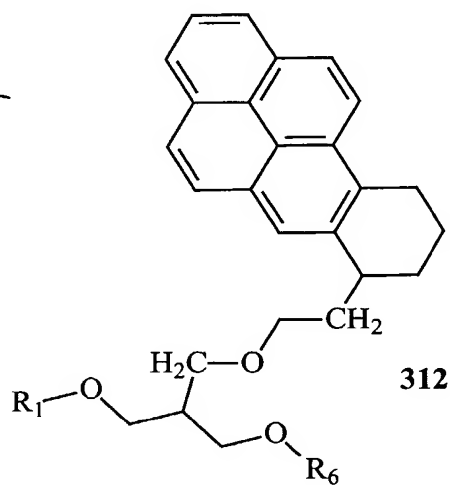
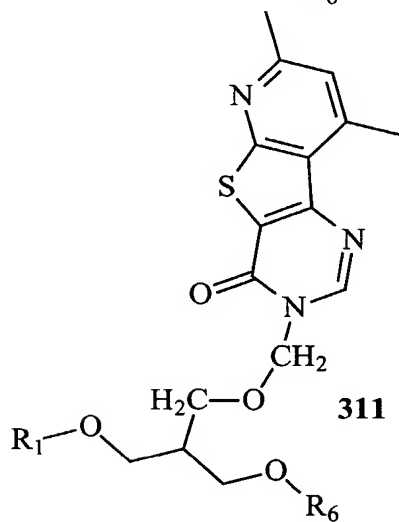
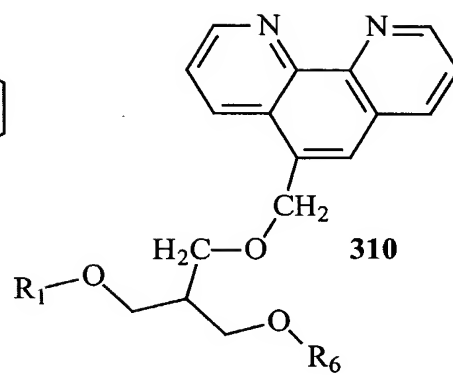
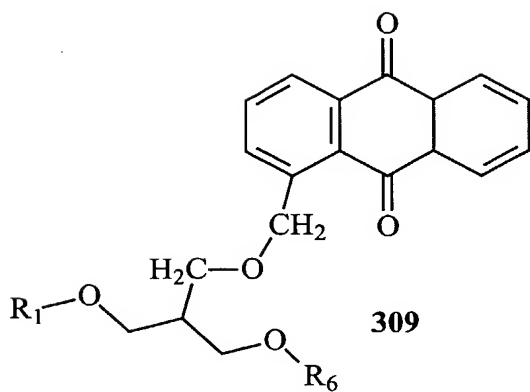
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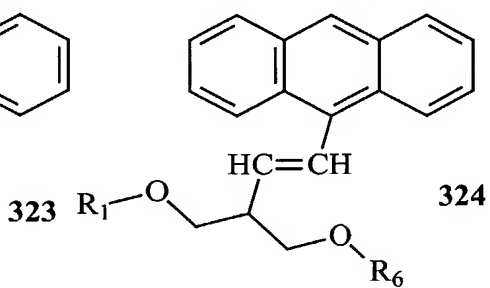
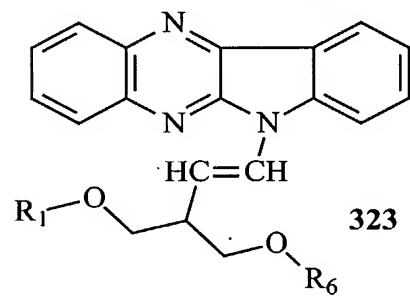
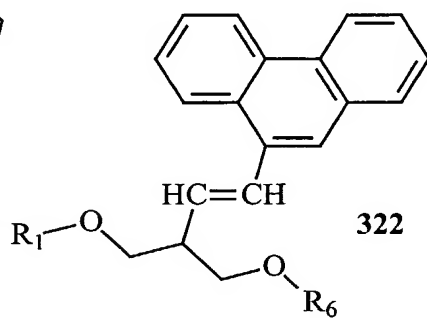
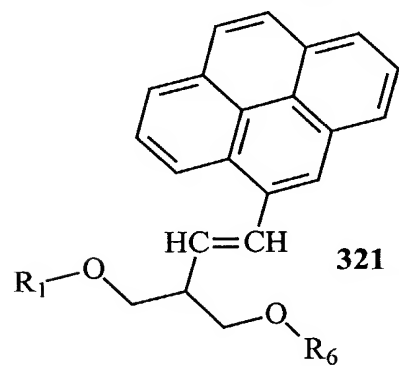
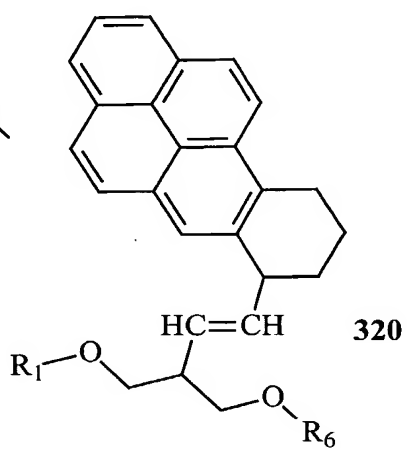
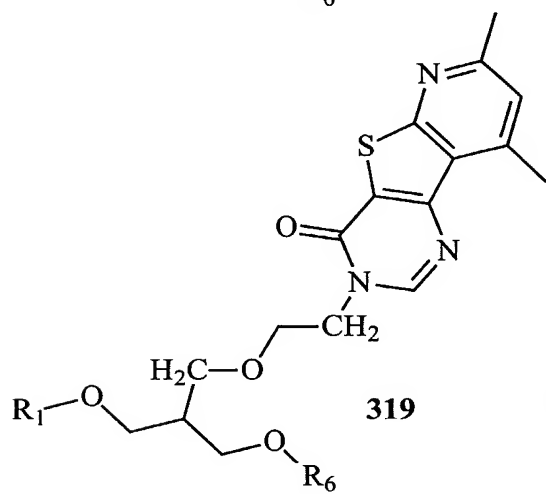
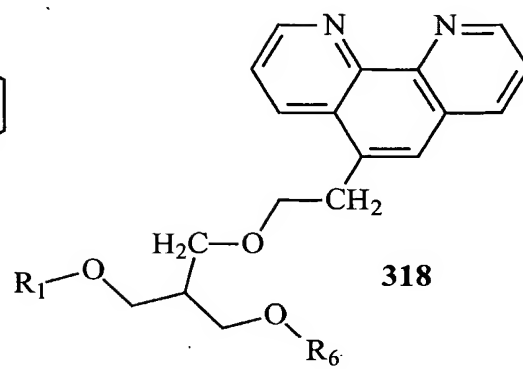
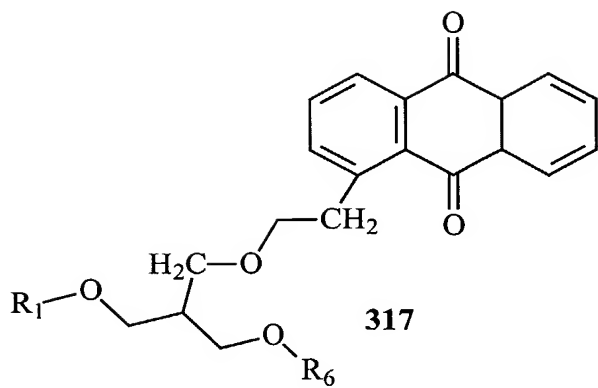


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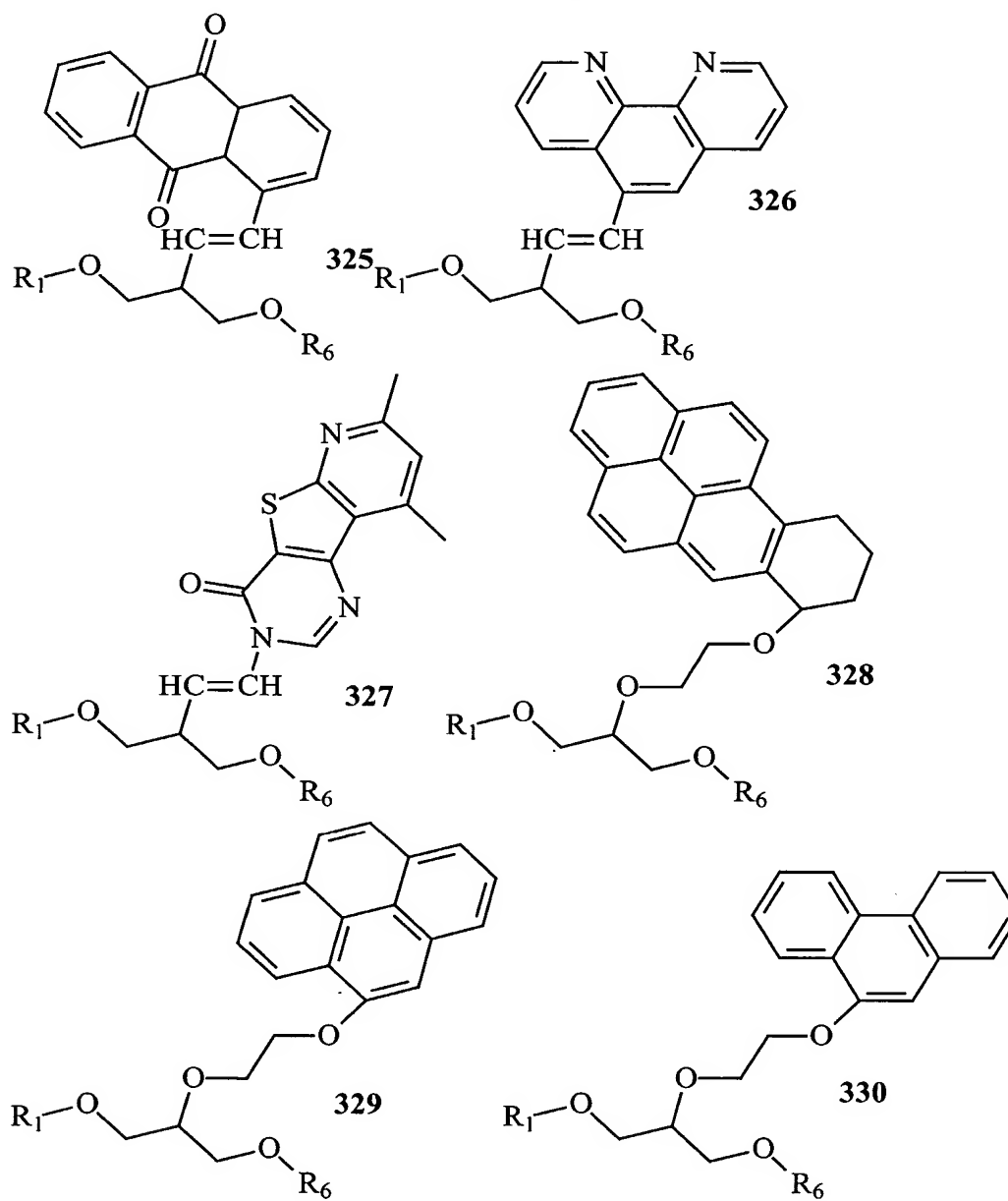


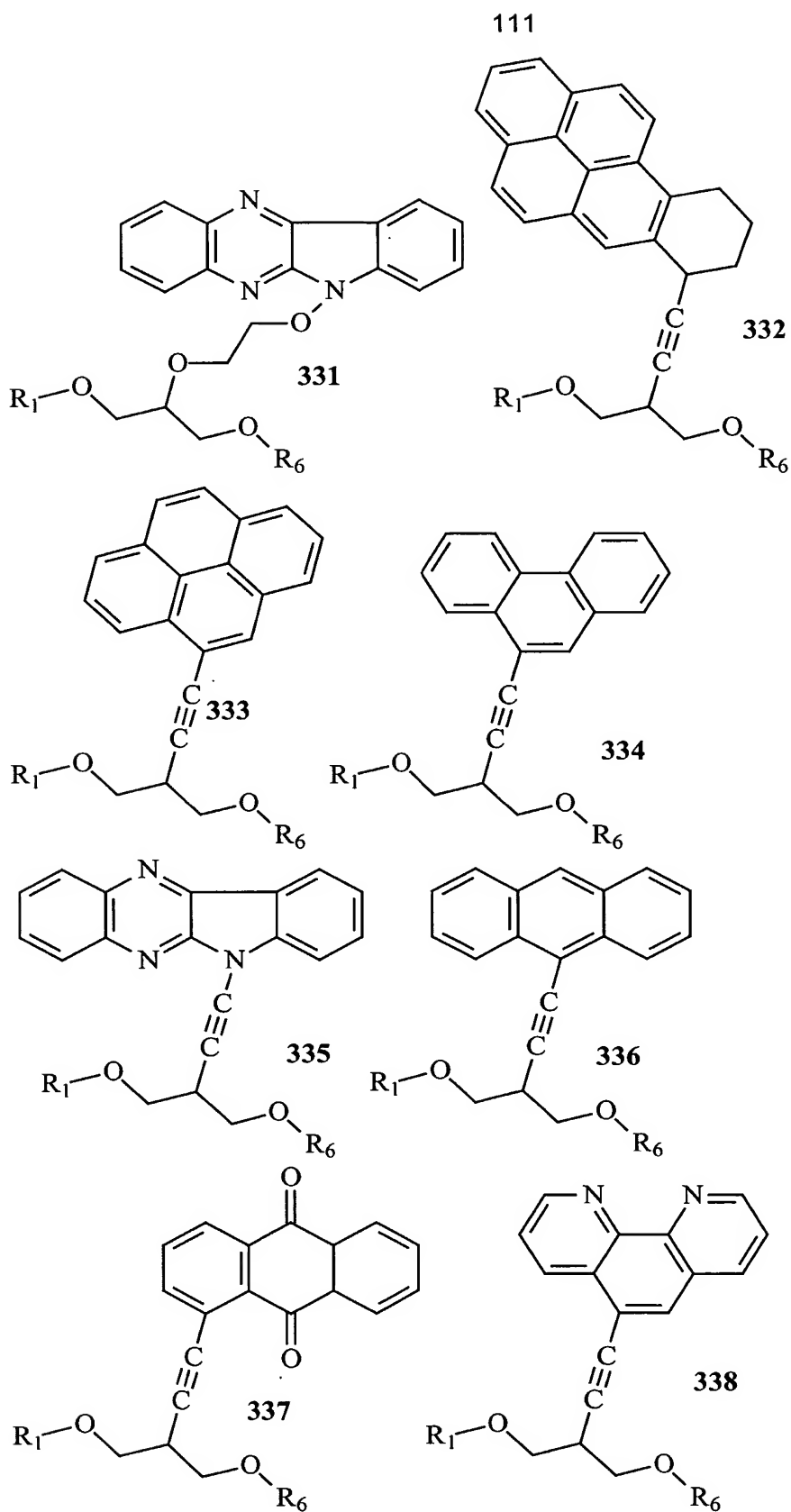


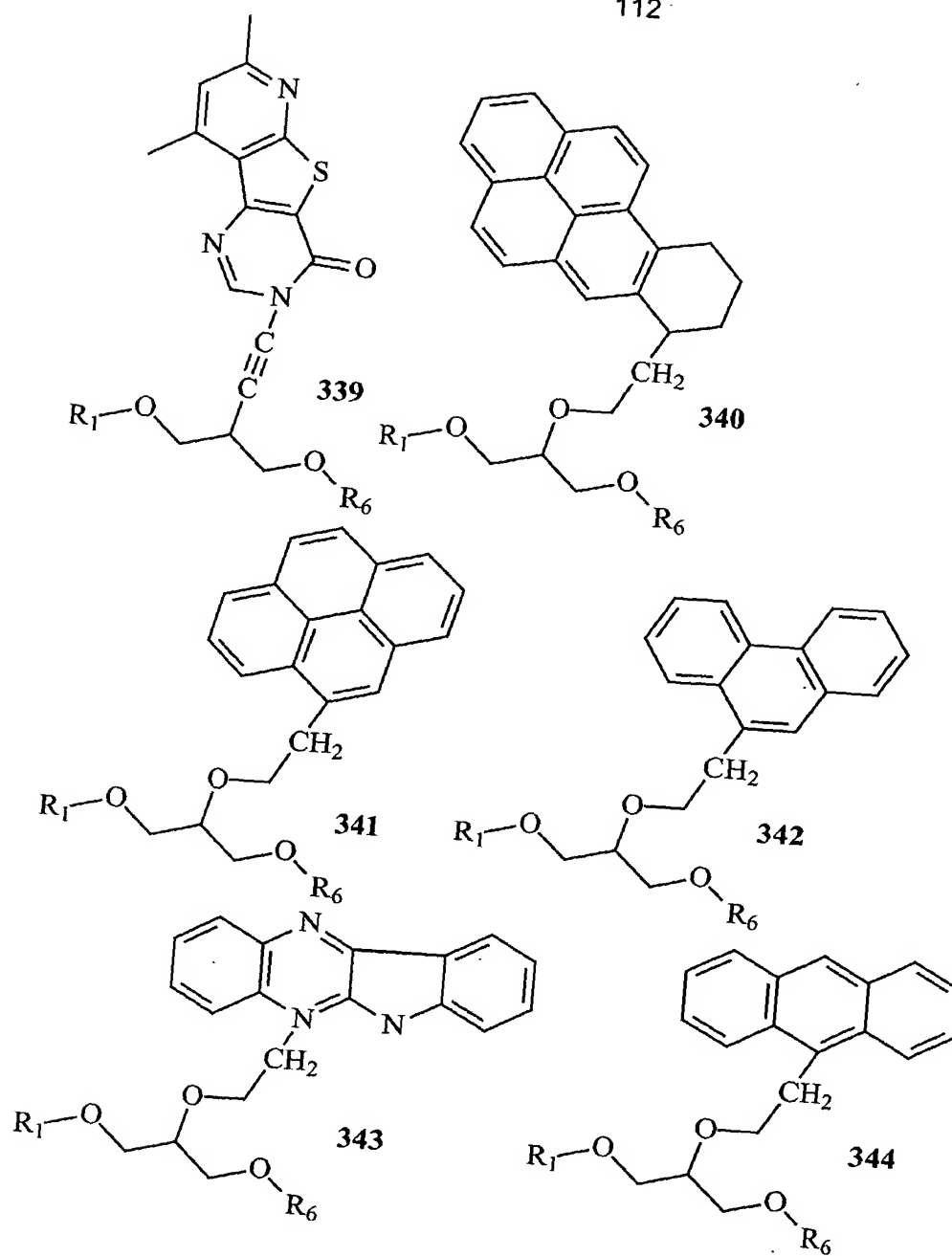
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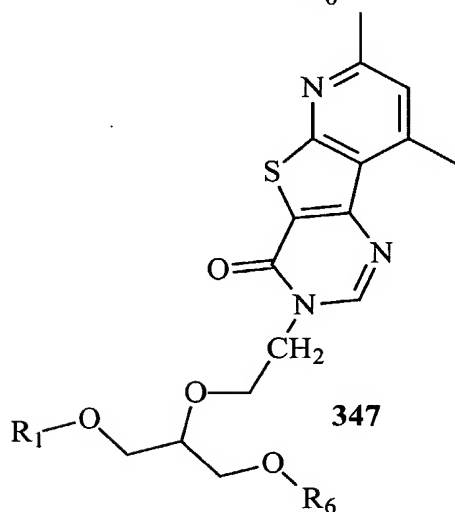
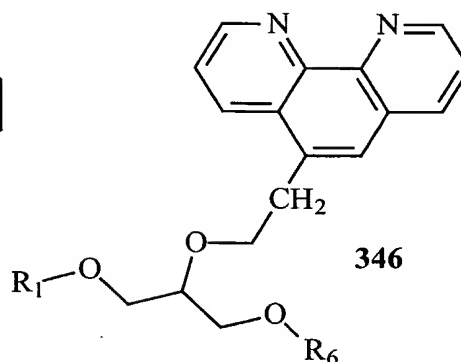
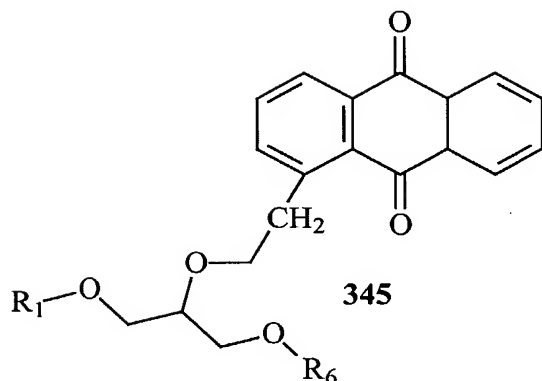
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5 wherein DMT and (CH₂CH₂CN) functions as protecting groups.

In one preferred embodiment of the present invention the intercalator pseudonucleotide is selected from the group consisting of phosphoramidites of 1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol. Even more preferably, the intercalator pseudonucleotide is selected from the group consisting of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol and the phosphoramidite of (R)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol.

Preparation of intercalator pseudonucleotides

15

The intercalator pseudonucleotides according to the present invention may be synthesised by any suitable method.

However preferably the method may comprise the steps of

5 a1) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group; and

10 b1) providing a linker precursor molecule comprising at least two reactive groups, said two reactive groups may optionally be individually protected; and

c1) reacting said intercalator with said linker precursor and thereby obtaining an intercalator-linker; and

15 d1) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked) and optionally comprising a linker part; and

20 e1) reacting said intercalator-linker with said backbone monomer precursor and obtaining an intercalator-linker-backbone monomer precursor;

or

25 a2) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked) and optionally comprising a linker part; and

30 b2) providing a linker precursor molecule comprising at least two reactive groups, said two reactive groups may optionally be individually protected; and

c2) reacting said monomer precursor unit with said linker precursor and thereby obtaining a backbone-linker; and

35 d2) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group; and

bases of a nucleic acid and optionally a linker part coupled to a reactive group;
and

5 e2) reacting said intercalator with said backbone-linker and obtaining an intercalator-linker-backbone monomer precursor;

or

10 a3) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and a linker part coupled to a reactive group; and

b3) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked), and a linker part; and

15

c3) reacting said intercalator-linker part with said backbone monomer precursor-linker and obtaining an intercalator-linker-backbone monomer precursor;

20 and

f) optionally protecting and/ or de-protecting said intercalator-linker-backbone monomer precursor; and

25 g) providing a phosphor containing compound capable of linking two pseudonucleotides, nucleotides and/ or nucleotide analogues together; and

h) reacting said phosphorous containing compound with said intercalator-linker-backbone monomer precursor; and

30

i) obtaining an intercalator pseudonucleotide

Preferably, the intercalator reactive group is selected so that it may react with the linker reactive group. Hence, if the linker reactive group is a nucleophil, then

35 preferably the intercalator reactive group is an electrophile, more preferably an

electrophile selected from the group consisting of halo alkyl, mesyloxy alkyl and tosyloxy alkyl. More preferably the intercalator reactive group is chloromethyl. Alternatively, the intercalator reactive group may be a nucleophile group for example a nucleophile group comprising hydroxy, thiol, selam, amine or mixture thereof.

5

Preferably, the cyclic or non cyclic alkane may be a polysubstituted alkane or alkoxy comprising at least three linker reactive groups. More preferably the polysubstituted alkane may comprise three nucleophilic groups such as, but not limited to, an alkane triole, an aminoalkan diol or mercaptoalkane diol. Preferably the polysubstituted

10 alkane contain one nucleophilic group that is more reactive than the others, alternatively two of the nucleophilic groups may be protected by a protecting group. More preferably the cyclic or non cyclic alkane is 2,2-dimethyl-4-methylhydroxy-1,3-dioxalan, even more preferably the alkane is D- α,β -isopropylidene glycerol .

15

Preferably, the linker reactive groups should be able to react with the intercalator reactive groups, for example the linker reactive groups may be a nucleophile group for example selected from the group consisting of hydroxy, thiol, selam and amine, preferably a hyhydroxy group. Alternatively the linker reactive group may be an electrophile group, for example selected from the group consisting of halogen,

20 triflates, mesylates and tosylates. In a preferred embodiment at least 2 linker reactive groups may be protected by a protecting group.

The method may furthermore comprise a step of attaching a protecting group to one or more reactive groups of the intercalator-precursor monomer. For example a DMT

25 group may be added by providing a DMT coupled to a halogen, such as Cl, and reacting the DMT-Cl with at least one linker reactive group. Accordingly, preferably at least one linker reactive group will be available and one protected. If this step is done prior to reaction with the phosphor comprising agent, then the phosphor comprising agent may only interact with one linker reactive group.

30

The phoshphor comprising agent may for example be a phosphoramidit, for example $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^i)_2$ or $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^i)_2\text{Cl}$. Preferably the phosphor comprising agent may be reacted with the intercalator-precursor in the presence of a base, such as $\text{N}(\text{et})_3$, $\text{N}(\text{'pr})_2\text{Et}$ and CH_2Cl_2 .

35

One specific non-limiting example of a method of synthesising an intercalator pseudonucleotide according to the present invention is outlined in example 1 and in figure 1.

5 Once the appropriate sequences of oligonucleotide or oligonucleotide analogue are determined, they are preferably chemically synthesised using commercially available methods and equipment: For example, the solid phase phosphoramidite method can be used to produce short oligonucleotide or oligonucleotide analogue comprising intercalator pseudonucleotides.

10

For example the oligonucleotides or oligonucleotide analogues may be synthesised by any of the methods described in "Current Protocols in Nucleic acid Chemistry" Volume 1, Beaucage et al., Wiley.

15 It is one objective of the present invention to provide methods of synthesising oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide, wherein synthesis may comprise the steps of

- 20 a) bringing an intercalator pseudonucleotide according to the present invention into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- b) reacting said intercalator pseudonucleotide with said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- c) optionally capping unreacted said support-bound oligonucleotide; and
- 25 d) optionally further elongating said oligonucleotide analogue by adding one or more nucleotides, nucleotide analogues or intercalator pseudonucleotides to the oligonucleotide analogue in a desired sequence; and
- e) cleaving said oligonucleotide analogue from said solid support; and
- f) thereby obtaining said oligonucleotide analogue comprising at least one intercalator pseudonucleotide.
- 30

In one embodiment of the present invention the synthesis may comprise the steps of

- 35 a. bringing an intercalator pseudonucleotide according to the invention comprising a reactive group, which may be protected by an acid la-

- bile protection group into contact with a growing chain of a support-bound oligonucleotide or oligonucleotide analogue; and
- b. reacting said intercalator pseudonucleotide with said support-bound oligonucleotide or oligonucleotide analogue; and
- 5 c. washing away excess reactants from product on the support; and
- d. optionally capping unreacted said support-bound oligonucleotide; and
- e. oxidizing the phosphite product to phosphate product; and
- f. washing away excess reactants from product on support; and
- g. optionally capping unreacted said support-bound oligonucleotide; and
- 10 h. repeating steps a)-g) until the desired number of intercalator pseudonucleotides are inserted; and
- i. optionally elongating said support-bound oligonucleotide containing at least one intercalator pseudonucleotide; and
- j. optionally repeating step a-i)
- 15 k. cleaving oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
- l. purifying oligonucleotide analogue containing acid labile protecting group; and
- m. removing acid labile protecting group with acidic media; and
- 20 n. obtaining a terminus pseudonucleotide modified oligonucleotide analogue containing at least one intercalator pseudonucleotide

In another embodiment of the present invention the synthesis may comprise the steps of

- 25 a) bringing an intercalator pseudonucleotide according to the present invention into contact with an universal support; and
- b) reacting said intercalator pseudonucleotide with the universal support; followed by step c) to j) as described in the method herein above.

30

It is also contained within the present invention that the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Subsequent purification of the oligonucleotide analogue is optional.

In yet another embodiment of the present invention the method comprises the synthesis an oligonucleotide or oligonucleotide analogue comprising at least one internally positioned intercalator pseudonucleotide, wherein synthesis may comprise the steps of

5

- a) bringing a nucleotide or nucleotide analogue protected with an acid labile protection group into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- 10 b) reacting the protected nucleotide analogue with the growing chain of said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- c) washing away excess reactants from product on support; and
- d) optionally capping unreacted said support-bound nucleotide; and
- 15 e) oxidizing the phosphite product to phosphate product; and
- f) washing away excess reactants from product on support; and
- g) optionally capping unreacted said support-bound nucleotide; and
- h) removing acid labile protecting group; and
- i) washing away excess reactants from product on the support; and
- 20 j) repeating steps a)-f) to obtain the desired oligonucleotide analogue sequence; and
- k) cleaving the oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
- l) purifying oligonucleotide containing acid labile protecting group; and
- 25 m) removing acid labile protecting group; and
- n) obtaining an intercalator modified oligonucleotide analogue.

30

Alternatively the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Purification of the oligonucleotide analogue is optional.

Excimer, exciplex, FRET and charge transfer

- An excimer is a dimer of compounds, which are associated in an electronic excited state, and which are dissociative in its ground state. When an isolated compound is excited it may lose its excitation or it may associate with another compound of the same kind_(which is not excited), whereby an excimer is formed. An excimer emits
5 fluorescence at a wavelength different from monomer fluorescence emission. When the excimer loses its excitation the association is no longer favourable and the two species will dissociate. An exciplex is an excimer like dimer, wherein the two compounds_are different.
- 10 Intramolecular excimers are formed by two moieties comprised within one molecule, for example 2 polyaromatic groups within the same molecule. Similar intramolecular exciplexes are formed by two moieties comprised within one molecule, for example by 2 different polyaromatic groups.
- 15 Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. FRET is dependent on the inverse 6th power of the intermolecular distance, making it useful over distances comparable with the dimension of biological
20 macromolecules. Preferably the donor and the acceptor must be in close proximity (typically between 10 to 100 Å) for FRET to occur. Furthermore, the absorption spectrum of the acceptor must overlap with the fluorescence emission spectrum of the donor. It is further preferred that the donor and the acceptor transition dipole orientations must be approximately parallel.
- 25 A charge transfer complex is a chemical complex in which there is weak coordination involving the transfer of charge between two intermolecular or intramolecular moieties, called an electron donor and an electron acceptor. These two moieties exhibit an observable charge-transfer absorption band during [formatting] charge-
30 transfer transition._[formatting]An example is phenoxinone, in which the phenol and quinone molecules are not held together by formal chemical bonds but are associated by transfer of charge between the compounds' aromatic ring systems.

Mutant sequences

The term "mutant sequence" according to the present invention covers a sequence which differs from a specific target sequence by at least one, such as 1, for example 2, such as 3, for example 4, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as from 10 to 20, for example from 20 to 50, such as more than 50 nucleobases. For example a mutant sequence according to the present invention may comprise one or more mutations.

The term "mutation" covers the change of one or more nucleotides for another one or more other nucleotides compared to a specific target sequence. Furthermore, the term "mutation" covers deletion and addition of nucleotides within a nucleic acid, for example deletion or addition of nucleotides compared to a target sequence. Additionally it covers the change in methylation pattern patterns.

In one embodiment the target sequence is a wild type sequence, i.e. the most frequently naturally occurring sequence, whereas the mutant sequence comprises one or more mutations compared to said wild type sequence. Accordingly, a mutation according to the present invention may in one embodiment be a polymorphism, such as a single nucleotide polymorphism (SNP). For example the polymorphism may be indicative of a specific DNA profile. Knowledge of a specific DNA profile may for example be employed to identify an individual. For example a specific DNA profile may be employed to identify a criminal or a potentially criminal or to identify a dead body or part of a dead body.

Furthermore, a specific DNA profile may be employed to determine relationship between individuals, for example parents-child relation ship or more distant relationships. Relationship may also be relationship between different species or different population of a given species.

In one embodiment the mutation may be indicative of a clinical condition or the mutation may be indicative of increased risk of a clinical condition.

Said clinical condition may for example be selected from the group consisting of neoplastic diseases, neurodegenerative diseases, cardiovascular diseases and metabolic disorders including diabetes.

- 5 Furthermore, the mutation may be indicative of a specific response to a predetermined drug treatment. For example, the mutation may be indicative of whether an individual will respond positively to said drug treatment or whether an individual can not tolerate a specific drug treatment.

10 Oligonucleotides comprising intercalator pseudonucleotides

One objective of the present invention is to provide oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide as described herein above. For example, the present invention relates to
15 oligonucleotides or oligonucleotide analogues synthesised by any of the methods described herein above or any other method known to the person skilled in the art.

High affinity of synthetic nucleic acids towards target nucleic acids may greatly facilitate detection assays and furthermore synthetic nucleic acids with high affinity
20 towards target nucleic acids may be useful for a number of other purposes, such as gene targeting and purification of nucleic acids. Oligonucleotides or Oligonucleotide analogues comprising intercalators have been shown to increase affinity for homologously complementary nucleic acids.

25 Accordingly it is an object of the present invention to provide oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide wherein the melting temperature of a hybrid consisting of said oligonucleotides or oligonucleotide analogues and a homologously complementary DNA (DNA hybrid) is significantly higher than the melting temperature of a hybrid between an
30 oligonucleotide or oligonucleotide analogue lacking intercalator pseudonucleotide(s) consisting of the same nucleotide sequence as said oligonucleotide or oligonucleotide analogue and said homologously complementary DNA (corresponding DNA hybrid).

Preferably, the melting temperature of the DNA hybrid is from 1 to 80°C, more preferably at least 2°C, even more preferably at least 5°C, yet more preferably at least 10°C higher than the melting temperature of the corresponding DNA hybrid.

5 The present invention may also provide oligonucleotides or oligonucleotide analogues comprising at least one internal intercalator pseudonucleotide. Positioning intercalator units internally allows for greater flexibility in design. Nucleic acid analogues comprising internally positioned intercalator pseudonucleotides may thus have higher affinity for homologously complementary nucleic acids than nucleic acid analogues that does not have internally positioned intercalator pseudonucleotides. Oligonucleotides or Oligonucleotide analogues comprising at least one internal intercalator pseudonucleotide may also be able to discriminate between RNA (including RNA-like nucleic acid analogues) and DNA (including DNA-like nucleic acid analogues). Furthermore internally positioned fluorescent intercalator monomers could find use in diagnostic tools.

For example such oligonucleotide analogues may comprise 1, such as 2, for example 3, such as 4, for example 5, such as from 1 to 5, such as, for example from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as more than 20 intercalatorpseudonucleotides.

In one embodiment the oligonucleotide or oligonucleotide analogue comprises at least 2 intercalator pseudonucleotides.

25 The intercalator pseudonucleotides may be placed in any desirable position within a given oligonucleotide or oligonucleotide analogue. For example, an intercalator pseudonucleotide may be placed at the end of the oligonucleotide or oligonucleotide analogue or an intercalator pseudonucleotide may be placed in an internal position within the oligonucleotide or oligonucleotide analogue.

30 When the oligonucleotide or oligonucleotide analogue comprise more than 1 intercalator pseudonucleotide, the intercalator pseudonucleotides may be placed in any position in relation to each other. For example they may be placed next to each other, or they may be positioned so that 1, such as 2, for example 3, such as 4, for example 5, such as more than 5 nucleotides are separating the intercalator

35

pseudonucleotides. In one preferred embodiment two intercalator pseudonucleotides within an oligonucleotide or oligonucleotide analogue are placed as next nearest neighbours, i.e. they can be placed at any position within the oligonucleotide or oligonucleotide analogue and having 1 nucleotide separating said two intercalator pseudonucleotides. In another preferred embodiment two intercalators are placed at or in close proximity to each end respectively of said oligonucleotide or oligonucleotide analogue.

The oligonucleotides or oligonucleotide analogues may comprise any kind of nucleotides and/or nucleotide analogues, such as the nucleotides and/or nucleotide analogues described herein above. For example, the oligonucleotides or oligonucleotide analogues may comprise nucleotides and/or nucleotide analogues comprised within DNA, RNA, LNA, PNA, ANA and HNA. Accordingly, the oligonucleotides or oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of PNA, Homo-DNA, b-D-Altopyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA, i.e. the oligonucleotide analogue may be selected from the group of PNA, Homo-DNA, b-D-Altopyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA and mixtures thereof.

One advantage of the oligonucleotides or oligonucleotide analogues according to the present invention is that the melting temperature of a hybrid consisting of an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide and an essentially complementary DNA (DNA hybrid) is significantly higher than the melting temperature of a duplex consisting of said essentially complementary DNA and a DNA complementary thereto.

Accordingly, oligonucleotides or oligonucleotide analogues according to the present invention may form hybrids with DNA with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, such as from 15°C to 20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C higher.

In particular, the increase in melting temperature may be achieved due to intercalation of the intercalator, because said intercalation may stabilise a DNA duplex. Accordingly, it is preferred that the intercalator is capable of intercalating between nucleobases of DNA. Preferably, the intercalator pseudonucleotides are placed as a bulge insertions or end insertions in the duplex (see herein below), which in some nucleic acids or nucleic acid analogues may allow for intercalation.

In one particular embodiment of the present invention the melting temperature of an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide and an essentially complementary RNA (RNA hybrid) or a RNA-like nucleic acid analogue (RNA-like hybrid) is significantly higher than the melting temperature of a duplex consisting of said essentially complementary RNA or RNA-like target and said oligonucleotide analogue comprising no intercalator pseudonucleotides. Preferably most or all of the intercalator pseudonucleotides of said oligonucleotide or oligonucleotide analogue are positioned at either or both ends.

Accordingly, oligonucleotides and/or oligonucleotide analogues according to the present invention may form hybrids with RNA or RNA-like nucleic acid analogues or RNA-like oligonucleotide analogues with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with from 2 to 20°C, for example from 5 to 15°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, such as from 15°C to 20°C or higher.

Said embodiment is particular in the sense that intercalator pseudonucleotides will preferably only stabilise towards RNA and RNA-like targets when positioned at the

end of said oligonucleotide or oligonucleotide analogue . This does however not exclude the positioning of intercalator pseudonucleotides in oligonucleotides or oligonucleotide analogues to be hybridised with RNA or RNA-like nucleic acid analogues such that said intercalator pseudonucleotides are placed in regions internal to the formed hybrid. This may be done to obtain certain hybrid instabilities or to affect the overall 2D or 3D structure of both intra- and inter-molecular complexes to be formed subsequent to hybridisation.

In another embodiment of the present invention an oligonucleotide and/or oligonucleotide analogue comprising one or more intercalator pseudonucleotides according to the present invention may form a triple stranded structure (triplex-structure) consisting of said oligonucleotide and/or oligonucleotide analogue bound by Hoogstein base pairing to a homologously complementary nucleic acid or nucleic acid analogue or oligonucleotide or oligonucleotide analogue.

In another preferred embodiment of the present invention said oligonucleotide or oligonucleotide analogue may increase the melting temperature of said Hoogstein base pairing in said triplex-structure.

In another even more preferred embodiment of the present invention said oligonucleotide or oligonucleotide analogue may increase the melting temperature of said Hoogstein base pairing in said triplex-structure in a manner not dependent on the presence of specific sequence restraints like purine-rich•pyrimidine-rich nucleic acid or nucleic acid analogue duplex target sequences. Accordingly, said Hoogstein basepairing in said triplex-structure has significantly higher melting temperature than the melting temperature of said Hoogstein basepairing to said duplex target if said oligonucleotide or oligonucleotide analogue had no intercalator pseudonucleotides.

Accordingly, oligonucleotides or oligonucleotide analogues according to the present invention may form triplex-structures with homologously complementary nucleic acid or nucleic acid analogue or oligonucleotide or oligonucleotide analogue with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with from 2-50°C, such as from 2-40°C, such as from 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, for example from 10°C to 15°C, such as from 15°C to

20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C.

5 In particular, the increase in melting temperature may be achieved due to intercalation of the intercalator, because said intercalation may stabilise a DNA triplex. Accordingly, it is preferred that the intercalator is capable of intercalating between nucleobases of a triplex-structure. Preferably, the intercalator
10 pseudonucleotide is placed as a bulge insertion in the duplex (see herein below), which in some nucleic acids or nucleic acid analogues may allow for intercalation.

Triplex-formation may or may not proceed in strand invasion, a process where the Hoogsteen base-paired third strand invades the target duplex and displaces part or all of the identical strand to form Watson-Crick base pairs with the complementary
15 strand. This can be exploited for several purposes.

The oligonucleotides and oligonucleotides according to the invention are suitably used for if only double stranded nucleic acid or nucleic acid analogue target is present and it is not possible, feasible or wanted to separate said target strands,
20 detection by single strand invasion of the region or double strand invasion of complementary regions, without prior melting of double stranded nucleic acid or nucleic acid analogue target, for triplex-formation and/or strand invasion.

Accordingly, in one embodiment of the present invention an oligonucleotide or
25 oligonucleotide analogue comprising at least one intercalator pseudonucleotide is provided that is able to invade a double stranded region of a nucleic acid or nucleic acid analogue molecule.

In a more preferred embodiment of the present invention an oligonucleotide or
30 oligonucleotide analogue comprising at least one intercalator pseudonucleotide that is able to invade a double stranded nucleic acid or nucleic acid analogue in a sequence specific manner is provided.

In a further embodiment of the present invention, said invading oligonucleotide
35 and/or oligonucleotide analogue comprising at least one intercalator

pseudonucleotide will bind to the complementary strand in a sequence specific manner with higher affinity than the strand displaced.

5 In one embodiment of the present invention the melting temperature of a hybrid consisting of an oligonucleotide analogue comprising at least one intercalator pseudonucleotide and a homologously complementary DNA (DNA hybrid), is significantly higher than the melting temperature of a hybrid consisting of said oligonucleotide or oligonucleotide analogue and a homologously complementary RNA (RNA hybrid) or RNA-like nucleic acid analogue target or RNA-like
10 oligonucleotide analogue target.

Said oligonucleotide may be any of the above described oligonucleotide analogues. For example, the oligonucleotide may be a DNA oligonucleotide (analogue) comprising at least one intercalator pseudonucleotide or a Homo-DNA, b-D-
15 Altopyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-
20 Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA oligonucleotide or mixtures hereof comprising at least one intercalator pseudonucleotide.

Accordingly, the affinity of said oligonucleotide or oligonucleotide analogue for DNA
25 is significantly higher than the affinity of said oligonucleotide or oligonucleotide analogue for RNA or an RNA-like target. Hence in a mixture comprising a limiting number of said oligonucleotide or oligonucleotide analogue and a homologously complementary DNA and a homologously complementary RNA or homologously complementary RNA-like target, the oligonucleotide or oligonucleotide analogue will
30 preferably hybridise to said homologously complementary DNA.

Preferably, the melting temperature of the DNA hybrid is at least 2°C, such as at least 5°C, for example at least 10°C, such as at least 15°C, for example at least 20°C, such as at least 25°C, for example at least 30°C, such as at least 35°C, for

example at least 40°C, such as from 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, for example from 10°C to 15°C, such as from 15°C to 20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C, for example from 50°C to 55°C, such as from 55°C to 60°C higher than the melting temperature of a homologously complementary RNA or RNA-like hybrid.

In a preferred embodiment of the present invention an oligonucleotide or oligonucleotide analogue containing at least one intercalator pseudonucleotide is hybridized to secondary structures of nucleic acids or nucleic acid analogues. In a more preferred embodiment said oligonucleotide or oligonucleotide analogue is capable of stabilizing such a hybridization to said secondary structure. Said secondary structures could be, but are not limited to stem-loop structures, Faraday junctions, fold-backs, H-knots, and bulges. In a special embodiment the secondary structure is a stem-loop structure of RNA, where an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide is designed in a way so said intercalator pseudonucleotide is hybridizing at the end of one of the three duplexes formed in the three-way junction between said secondary structure and said oligonucleotide or oligonucleotide analogue.

Solid supports

In one preferred embodiment of the present invention the oligonucleotide analogues according to the present invention or target nucleic acids are coupled to a solid support. The separation of oligonucleotide analogues together with nucleic acids or nucleic acid analogues hybridized to said oligonucleotide analogues from the mixture might then be performed by separating said solid support from the mixture.

Many different kinds of solid supports are suitable for the method, depending of the desired outcome.

In one embodiment the solid support is an activated surface. An activated surface facilitates coupling of the oligonucleotides or oligonucleotide analogues to the solid support.

- 5 The solid support may for example be selected from the group consisting of magnetic beads, alumina beads agarose beads, sepharose beads, glass, plastic surfaces, heavy metals and chips surfaces.

10 Magnetic beads include beads comprising a magnetic material that allow the beads to be separated from a suspension using a magnet. Alumina beads include barcoded beads that allows the bead to be recognised.

15 Agarose beads and sepharose beads may for example be separated from a suspension by centrifugation or filtration.

20 Plastic surfaces include for example microtiter plates or other plastic devices that may be suitable for example for diagnosis.

25 Chip surfaces may be made of any suitable materials, for instance, glass, resin, metal, glass covered with polymer coat, glass covered with metal coat and resin covered with metal coat. Also employable is a SPR (surface plasmon resonance) sensor plate, which is described in Japanese Patent Provisional Publication No. 11-332595. CCD is also employable as described in Nucleic Acids Research, 1994, Vol. 22, No. 11, 2124-2125.

30 Chip surfaces include small polyacrylamide gels on a glass plate where to oligonucleotides or oligonucleotide analogues may be fixed by making a covalent bond between the polyacrylamide and the oligonucleotide (Yershov, G., et al., Proc. Natl. Acad. Sci. USA, 94, 4913(1996)).

35 Chip surfaces may also be silica chips as described by Sosnowski, R. G., et al., Proc. Natl. Acad. Sci. USA, 94, 1119-1123 (1997). Such chips are prepared by a process comprising the steps of placing an array of microelectrodes on a silica chip, forming on the microelectrode a streptavidin-comprising agarose layer, and

attaching biotin-modified DNA fragments to the agarose layer by positively charging the agarose layer.

Furthermore, chip surfaces may be prepared as described by Schena, M., et al.,
5 Proc. Natl. Acad. Sci. USA, 93, 10614-10619 (1996) wherein a process comprising the steps of preparing a suspension of an amino group-modified PCR product in SSC (i.e., standard sodium chloride-citric acid buffer solution), spotting the suspension onto a slide glass, incubating the spotted glass slide, treating the incubated slide glass with sodium boronhydride, and heating thus treated slide
10 glass.

Further more columns containing the solid material can be used.

Sample material description

15 The present invention provides methods for detecting nucleic acid or nucleic acid analogue comprising a specific target sequence as well as methods to differentiate between nucleic acid or nucleic acid analogue comprising a specific target sequence and nucleic acids comprising a mutant sequence. Said target sequence may be de-
20 tected in any useful mixtures comprising nucleic acids and/or nucleic acid analogues.

The mixture may be comprised within a cell, for example within an intact cell. The cell may for example be a prokaryotic cell or an eukaryotic cell, such as a plant cell
25 or a mammalian cell. In such an embodiment the method may be employed for in situ hybridization.

The test nucleic acid or nucleic acid analogue sample may for example be a synthetically prepared sample, which may or may not have been further processed in
30 vitro. The test nucleic acid or nucleic acid analogue sample may comprise any nucleic acid or nucleic acid analogue, for example DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-

DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA and mixtures thereof and hybrids thereof.

5 Frequently, it is desirable to test the DNA or RNA of an individual, such as a mammal, for example a human being. In that case the test nucleic acid or nucleic acid analogue sample is a sample derived from said individual. The sample may be derived from a body fluid sample for example a blood sample, a biopsy, a sample of hair, nails or the like or any other suitable sample.

10

The sample may be processed in vitro prior to detection of the presence of corresponding target nucleic acids and/ or nucleic acid analogues and/ or the mutants hereof. For example the sample may be subjected to one or more purification steps that may purify nucleic acids from the sample completely or partially. Furthermore, the sample may have been subjected to amplification steps, wherein the amount of nucleic acids have been amplified, for example by polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), ligase chain reaction or any other suitable amplification process.

15

20 In one preferred embodiment of the present invention the test nucleic acid sample is selected from the group consisting of genomic DNA or an amplification product of genomic DNA, such as a PCR amplification product of genomic DNA.

25

The method may involve a separation step prior to detection, wherein hybridized oligonucleotide or oligonucleotide analogue is separated from unhybridised oligonucleotide or oligonucleotide analogue, which may facilitate specific detection of only hybridized oligonucleotide or oligonucleotide analogue. For example, the mixture of nucleic acids may be immobilized on a solid support prior to hybridization with the oligonucleotide or oligonucleotide analogue. After hybridization, unhybridised oligonucleotide or oligonucleotide analogue may be washed away and hybridized oligonucleotide or oligonucleotide analogue may be detected.

30

Alternatively, the method may involve the method of separation of sequence specific DNA(s) from a mixture as outlined herein above, prior to detection. For example, the oligonucleotide or oligonucleotide analogue may be bound to a solid support and

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after hybridization unbound nucleic acids, may be washed away and bound nucleic acids may be detected.

5 The target DNA may for example be a particular gene, a gene segment, a microsatellite or any other DNA sequence. Of particular interest is the detection of particular DNAs, which may be of eukaryotic, prokaryotic, Archae or viral origin. Importantly, the invention may assist in the diagnosis and/or genotyping of various infectious diseases by assaying for particular sequences known to be associated with a particular microorganism. The target DNA may be provided in a complex biological mixture of
10 nucleic acid (RNA and DNA) and non-nucleic acids, for example an intact cell or a crude cell extract.

If the target DNA is double stranded or otherwise have significant secondary and tertiary structure, it may need to be heated prior to hybridization. In this case, heating
15 may occur prior to or after the introduction of the nucleic acids into the hybridization medium containing the oligonucleotide analogue. It may also be desirable in some cases to extract the nucleic acids from the complex biological samples prior to the hybridization assay to reduce background interference by any methods known in the art.

20 The hybridization and extraction methods of the present invention may be applied to a complex biological mixture of nucleic acid (DNA and/or RNA) and non-nucleic acids. Such a complex biological mixture includes a wide range of eukaryotic and prokaryotic cells, including protoplasts; or other biological materials that may harbor
25 target deoxyribonucleic acids. The methods are thus applicable to tissue culture animal cells, animal cells (e.g., blood, serum, plasma, reticulocytes, lymphocytes, urine, bone marrow tissue, cerebrospinal fluid or any product prepared from blood or lymph) or any type of tissue biopsy (e.g. a muscle biopsy, a liver biopsy, a kidney biopsy, a bladder biopsy, a bone biopsy, a cartilage biopsy, a skin biopsy, a pancreas biopsy, a biopsy of the intestinal tract, a thymus biopsy, a mammal biopsy, an
30 uterus biopsy, a testicular biopsy, an eye biopsy or a brain biopsy, homogenized in lysis buffer), plant cells or other cells sensitive to osmotic shock and cells of bacteria, yeasts, viruses, mycoplasmas, protozoa, rickettsia, fungi and other small microbial cells and the like. The assay and isolation procedures of the present invention
35 are useful, for instance, for detecting non-pathogenic or pathogenic microorganisms

of interest. By detecting specific hybridization between oligonucleotides or oligonucleotide analogues comprising intercalator pseudonucleotide(s) and nucleic acids resident in the biological sample, the presence of the microorganisms may be established.

5

Detection and differentiating between target nucleic acids, target nucleic acid analogues and mutants sequences by melting temperature

10 The present invention relates to oligonucleotide analogues comprising at least one intercalator pseudonucleotide.

In one embodiment of the present invention said oligonucleotide analogue has a significantly higher affinity for its target nucleic acid sequence, than for any other nucleic acid sequences present in the mixture.

15

In a preferred embodiment of the present invention, the detection procedure is dependent on temperature, including assays where washing procedures are used to remove nucleic acids or nucleic acid analogues with a lower affinity for said oligonucleotide analogue than the target nucleic acid or target nucleic acid analogue has.

20

In another preferred embodiment of the present invention high melting temperature indicates the presence of target nucleic acid in the mixture.

25 In an even more preferred embodiment the detection of hybridization is carried out after stringent washing procedures and a positive signal indicates the presence of target nucleic acid in the mixture.

30 The determination of the extent of hybridization may be carried out by any of the methods well known in the art. The oligonucleotide analogues, comprising intercalator pseudonucleotides according to the present invention, may be used to detect hybridization directly. In addition the oligonucleotide analogues, comprising intercalator pseudonucleotides according to the present invention, may be coupled to one or more detectable labels. The most common methods of detection are the

use of ligands that bind to labeled antibodies, fluorophores or chemiluminescent agents.

In one embodiment of the present invention, several non-identical oligonucleotides and/or oligonucleotide analogues comprising intercalator pseudonucleotides according to this invention may be used at one time to address different target nucleic acids or nucleic acid analogues in a mixture, thus facilitating the detection of a number of nucleic acids or nucleic acid analogues corresponding to the number of said oligonucleotides and/or oligonucleotide analogues.

Detection and differentiating between target nucleic acids, target nucleic acid analogues and the mutants sequences using spectral properties of intercalator pseudonucleotides

The present invention relates to oligonucleotide analogues comprising at least one intercalator pseudonucleotide,

wherein said oligonucleotide analogue comprises monomer fluorescence and/ or intramolecular excimer and/ or intramolecular exciplex and/ or intramolecular FRET complex and/ or an intramolecular charge transfer complex.

In a preferred embodiment an oligonucleotide analogue comprise at least two intercalator pseudonucleotides, and said intercalator pseudonucleotides are capable of forming an excimer and/ or an exciplex and/ or a charge-transfer and/ or a FRET complex. Preferably said two intercalators of an oligonucleotide or oligonucleotide analogue, is placed in a distance from one another within the oligonucleotide or oligonucleotide analogue so they can interact and hence form an intramolecular excimer, an intramolecular exciplex, a FRET complex or a charge transfer complex.

Detection of a mismatched base pair in a hybrid between a target nucleic acid and an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide according to the present invention can be done in a region within n nucleobases to each side of any intercalator pseudonucleotide. n is selected from the group consisting of integers in the range from 1 to 3.

Hence the intercalator pseudonucleotide comprising oligonucleotide analogue sequence should be selected according to the mutation, which should be detected. Accordingly, said oligonucleotide or oligonucleotide analogue preferably is capable of hybridizing with the sequences flanking the n nucleotides around any of the pseudonucleotides involved in the detection of mutants.

Accordingly, it is preferred that said oligonucleotide or oligonucleotide analogue comprises sequences that can hybridize with the nucleic acid sequence, which might be mutated, or the mutated nucleic acid sequence as well as sequences that can hybridize with sequences flanking said nucleic acid sequences in a wild type individual.

In one preferred embodiment of the present invention said oligonucleotide analogue comprises a sequence complementary to the nucleic acid sequence, and said oligonucleotide analogue furthermore comprises one intercalator pseudonucleotide inserted at both sides of said complementary sequence.

Alternatively, the oligonucleotide analogue comprises a sequence complementary to the mutated nucleic acid sequence and said oligonucleotide analogue furthermore comprises one intercalator pseudonucleotide inserted at both sides of said complementary sequence.

In another preferred embodiment of the present invention the intercalators of at least two intercalator pseudonucleotides within an oligonucleotide or oligonucleotide analogue are capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex and/ or an intramolecular charge transfer complex, when at least one of the n nucleotides at either side of any of said intercalator pseudonucleotides as described above is unhybridised.

More preferably the intercalators of at least two intercalator pseudonucleotides within an oligonucleotide analogue are capable of forming an intramolecular excimer and/ or an intramolecular exciplex and/ or an intramolecular FRET complex and/ or an intramolecular charge transfer complex, when at least one of the basepairs comprised within the n nucleotides at either side of any of said intercalator

pseudonucleotides is unhybridised and said intercalators are not capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex or an intramolecular charge transfer complex, when all the n nucleotides at either side of any of said intercalator pseudonucleotides are hybridized.

5

In another preferred embodiment of the present invention the intercalators of at least two intercalator pseudonucleotides within two oligonucleotides and/ or oligonucleotide analogues are capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex and/ or an intermolecular charge transfer complex, when they are hybridised to consecutive sequences of a target nucleic acid or nucleic acid analogue and at least one of the n nucleotides at either side of any of said intercalator pseudonucleotides as described above is unhybridised.

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15

More preferably the intercalators of said at least two intercalator pseudonucleotides are capable of forming an intermolecular excimer and/ or an intermolecular exciplex and/ or an intermolecular FRET complex and/ or an intermolecular charge transfer complex, when at least one of the basepairs comprised within the n nucleotides at either side of any of said intercalator pseudonucleotides is unhybridised and said intercalators are not capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex or an intramolecular charge transfer complex, when all the n nucleotides at either side of any of said intercalator pseudonucleotides are hybridized.

20

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In another preferred embodiment of the present invention the intercalators of an oligonucleotide analogue according to the present invention comprising at least one intercalator pseudonucleotide are capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex and/ or an intermolecular charge transfer complex, when said oligonucleotide analogue is hybridized to its corresponding target nucleic acid or nucleic acid analogue.

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More preferably the intercalators of an oligonucleotide or oligonucleotide analogue according to the present invention comprising at least one intercalator pseudonucleotide are capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex and/ or an intermolecular charge transfer

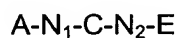
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complex, when said oligonucleotide or oligonucleotide analogue is hybridized to its complementary target nucleic acid or nucleic acid analogue and said oligonucleotide or oligonucleotide analogue comprising said intercalator(s) are not capable of hybridizing to a mutated sequence, often under high stringency conditions, of the target nucleic acid or nucleic acid analogue and hence not capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex or an intermolecular charge transfer complex.

It is only possible for two molecular moieties to form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex if the two molecules are positioned in relation to one another, so that they might interact with one another.

Intercalators according to the present invention are capable of co-stacking with nucleobases. When oligonucleotide analogues comprising said intercalators hybridize with corresponding DNA, said intercalators will preferably co-stack with the nucleobases of the hybrid. If all n nucleobases around each of the at least two intercalators form matched base-pairs this will preferably result in a steric hindrance of the intercalator moieties, so that said intercalators will not be able to interact and accordingly not be able to form an intramolecular excimer, an intramolecular exciplex, FRET complex or a charge transfer complex.

Accordingly, the most preferred oligonucleotide or oligonucleotide analogues to be used with the present invention are oligonucleotide or oligonucleotide analogues of the structure



wherein A is a sequence essentially complementary to one sequence directly flanking the potential mutation, preferably A is complementary to one sequence directly flanking the potential mutation; and

E is a sequence essentially complementary to the other sequence directly flanking the potential mutation, preferably E is complementary to the other sequence directly flanking the potential mutation; and

N_1 and N_2 are intercalator pseudonucleotides, which may or may not be identical; and

5 C is a sequence complementary to the target sequence but not complementary to the mutant sequence or C is a sequence complementary to the mutant sequence but not the target sequence; and

Alternatively a preferred oligonucleotide according to the invention has the structure

10 A-B- N_1 -C- N_2 -D-E

Wherein A, C, E, N_1 and N_2 are as defined herein above and

15 B is a sequence complementary to the target sequence but not complementary to the mutant sequence or B is a sequence complementary to the mutant sequence but not the target sequence; and

20 D is a sequence complementary to the target sequence but not complementary to the mutant sequence or D is a sequence complementary to the mutant sequence but not the target sequence.

Hence, when these most preferred oligonucleotides or oligonucleotide analogues are hybridized to their complementary sequences, the intercalator pseudonucleotides will be present as bulges.

25 The sequence of the oligonucleotide analogue according to the present invention should be chosen so that the mutant to test for is placed within sequences B, C or D. In one preferred embodiment the mutation is a single point mutation, such as a SNP (see herein below) and accordingly B, C and D are only 1 nucleotide long and
30 the intercalator pseudonucleotides are positioned as next nearest neighbors.

A and E may individually be any useful length, such as 2 to 5, for example 5 to 10, such as 10 to 15, for example 15 to 20, such as 20 to 30, for example more than 30
35 nucleotides long.

Detection of a target nucleic acid or nucleic acid analogue may also be carried out using the spectral properties of monomer pseudonucleotide units like for example fluorescence then, the at least one intercalator pseudonucleotide can be positioned in any relative position to each other according to the present invention.

5

Method of detecting the presence of corresponding target nucleic acids and/ or target nucleic acid analogues and the mutants hereof

10 It is one object of the present invention to provide methods of detecting a nucleic acid or nucleic acid analogue comprising a specific target sequence, which may differ from any other sequences by at least one nucleobase, wherein the method comprises the steps of

- 15 a) providing a mixture of nucleic acids and/ or nucleic acid analogues, which is desirable to test for the mutation; and
- b) providing an oligonucleotide analogue comprising at least one intercalator pseudonucleotide capable of hybridising with said specific sequence; and
- c) incubating the oligonucleotide analogue with the mixture comprising nucleic acids or nucleic acid analogues under conditions allowing for hybridisation;
- 20 and
- d) washing away sequences that have less affinity to said oligonucleotide analogue than the target sequence; and
- e) determining the presence or absence the target sequence.

25

Furthermore, the invention provides methods of differentiating between a nucleic acid or nucleic acid analogue comprising a specific target sequence and a nucleic acid comprising a mutant sequence.

30

Preferably, either hybridization or separation is carried out under high-stringency conditions. For example separation in solution may be done e.g. by electrophoresis or chromatography.

More preferably there is only hybridization between the oligonucleotide or oligonucleotide analogue, comprising at least one intercalator pseudonucleotide according to the present invention, and the corresponding target nucleic acid and/ or nucleic acid analogue when hybridization is carried out under high-stringency conditions.

In a preferred embodiment detection of hybridization is carried out by the use of a label. The label may be coupled to either the oligonucleotide analogue according to the present invention or to the target nucleic acid and/ or nucleic acid analogue or both or the label may be free in solution.

In one embodiment of the present invention either the oligonucleotide analogues according to the present invention or nucleic acids and/ or nucleic acid analogues may be affixed to a solid support. Separation is then typically done by one or more washing steps under high-stringency conditions.

In a preferred embodiment of the present invention either the oligonucleotide oligonucleotide or analogues according to the present invention or nucleic acids and/ or nucleic acid analogues may be affixed to a solid support, for example a chip surface, thus allowing for the simultaneous detection of many hybridization assays in parallel.

In one embodiment, detection of the presence of hybrids between target nucleic acids and/ or target nucleic acid analogues and said corresponding oligonucleotide analogues is carried out by the use of unspecific and/or small molecule [mening: ikke bundne til oligoer] stains for double stranded nucleic acids or double stranded nucleic acid derivatives.

In another preferred embodiment, detection of the presence of hybrids between target nucleic acids and/ or target nucleic acid analogues and the corresponding oligonucleotide analogues is carried out by intermolecular excimer, exciplex, FRET and/ or charge-transfer complex formation.

In yet another preferred embodiment, an oligonucleotide analogue comprising at least one intercalator pseudonucleotide according to the present invention is complementary to the target nucleic acid and/ or the target nucleic acid analogue.

5 In still another preferred embodiment, an oligonucleotide analogue comprising at least one intercalator pseudonucleotide according to the present invention is complementary to the mutant of the target nucleic acid and/ or the target nucleic acid analogue

10 In a further preferred embodiment, oligonucleotide analogues comprising at least one intercalator pseudonucleotide according to the present invention are individually complementary to either the target nucleic acid and/ or the target nucleic acid analogue or the mutants hereof.

15 In the most preferred embodiment more oligonucleotide analogues comprising at least one intercalator pseudonucleotide according to the present invention are used to differentiate between target nucleic acid and/ or the target nucleic acid analogue and known types of single point mutations hereof.

20 In a preferred embodiment differentiation between target nucleic acids and/ or target nucleic acid analogues and the mutants hereof is carried out by the use of intermolecular excimers, exciplexes, FRET complexes and/ or charge-transfer complexes. Hence it is a preferred embodiment to use labeled nucleic acids and/ or nucleic acid analogues together with labeled oligonucleotide analogues to create
25 said intermolecular excimers, exciplexes, FRET complexes and/ or charge-transfer complexes, with the proviso that either the labeled nucleic acids and/ or nucleic acid analogues or the labeled oligonucleotide analogues or both comprise at least one intercalator pseudonucleotide according to the present invention. In an even more preferred embodiment at least one of the labels is an intercalator
30 pseudonucleotide.

In another preferred embodiment differentiation between target nucleic acids and/ or target nucleic acid analogues and the mutants hereof is carried out by the use of
35 intermolecular excimers, exciplexes, FRET complexes and/ or charge-transfer

complexes. Hence it is a preferred embodiment to use labeled signal oligonucleotides or oligonucleotide analogues together with an oligonucleotide analogues (catching probe) to create said intermolecular excimers, exciplexes, FRET complexes and/ or charge-transfer complexes, with the proviso that either the labeled signal oligonucleotides or oligonucleotide analogues or said catching probe or both comprise at least one intercalator pseudonucleotide according to the present invention. In an even more preferred embodiment at least one of the labels is an intercalator pseudonucleotide.

Method for detection including enzymatic step

The presence or absence of a target sequence and/or mutant sequence according to the present invention may be performed by a method including an enzymatic step.

Accordingly, in one embodiment the present invention relates to methods for detecting a target sequence and/or a mutant sequence, which differ from the target sequence by at least one nucleobase, preferably which differ from the target sequence by in the range from 1 to 5 nucleobases.

A more preferred embodiment of the present invention relates to methods for detecting a target sequence and/or a mutant sequence, which differ from the target sequence at least at one nucleobases position [skal kunne dække over forskellig metyleringsgrad, ligesom variation i sekvenserne. Var bange for at den anden sætning ovenfor kun galdt ved deletion og addition], which comprises the steps of

- a) providing a mixture of nucleic acids and/ or nucleic acid analogues, which is desirable to test for the presence of target sequences or a mutant sequence; and
- b) providing an oligonucleotide or oligonucleotide analogue as described herein above, wherein said oligonucleotide analogue is capable of hybridizing with said target sequence and/or the mutant sequence; and

- c) incubating said oligonucleotide or oligonucleotide analogue with said mixture of nucleic acids and/ or nucleic acid analogues under conditions allowing for hybridization; and
- d) using said hybridized target sequence for templating extension of the 3' end of said oligonucleotide analogue with a label or labeled nucleotide or nucleotide analogue or oligonucleotide or oligonucleotide analogue; and
- e) optionally separating hybridized and unhybridized sequences; and
- f) determining the presence or absence of the target sequence and/or mutant sequence.

Preferably, hybridization and separation are carried out under high-stringency conditions. For example separation in solution may be done e.g. by electrophoresis or chromatography.

More preferably there is only hybridization between the oligonucleotide or oligonucleotide analogue, comprising at least one intercalator pseudonucleotide according to the present invention, and the corresponding target nucleic acid and/ or nucleic acid analogue when hybridization is carried out under high-stringency conditions.

Preferably detection of hybridization is carried out by the use of a label attached to a nucleotide, nucleotide analogue, oligonucleotide or oligonucleotide analogue that are added to the 3' end of an oligonucleotide analogue according to the present invention. The addition of a labeled nucleotide or nucleotide analogue is preferably done enzymatically e.g. by DNA polymerases. Addition of a labeled oligonucleotide or oligonucleotide analogue is preferably done enzymatically e.g. by a ligase. Prior to coupling of the label, it may be free in solution, bound to nucleotides or nucleotide analogues or oligonucleotides or oligonucleotide analogues or other components that may or may not be a part of the complex to be detected.

In one embodiment of the present invention either the oligonucleotide or oligonucleotide analogues according to the present invention or nucleic acids and/ or nucleic acid analogues may be affixed to a solid support. Separation is then typically done by one or more washing steps under high-stringency conditions.

5 In a preferred embodiment of the present invention either the oligonucleotide or oligonucleotide analogues according to the present invention or nucleic acids and/or nucleic acid analogues may be affixed to a solid support, for example a chip surface, thus allowing the simultaneous detection of many hybridization assays in parallel.

10 In yet a preferred embodiment, a signal probe comprising a label is added prior to determination of hybridization. Said signal probe is preferably complementary to a region of the target nucleic acid or nucleic acid analogue that is right next to hybridization region of the oligonucleotide analogue according to the present invention. The signal probe may or may not comprise intercalator pseudonucleotides.

15 In another preferred embodiment, detection of the presence of hybrids between target nucleic acids and/or target nucleic acid analogues and the corresponding oligonucleotide analogues is carried out by intermolecular excimer, exciplex, FRET and/or charge-transfer complex formation.

20 In yet another preferred embodiment, an oligonucleotide analogue comprising at least one intercalator pseudonucleotide according to the present invention is complementary to the target nucleic acid and/or the target nucleic acid analogue.

25 In still another preferred embodiment, an oligonucleotide analogue comprising at least one intercalator pseudonucleotide according to the present invention is complementary to the mutant of the target nucleic acid and/or the target nucleic acid analogue.

30 In a further preferred embodiment, oligonucleotide analogues comprising at least one intercalator pseudonucleotide according to the present invention are individually complementary to either the target nucleic acid and/or the target nucleic acid analogue or the mutants hereof.

In the most preferred embodiment more oligonucleotide analogues comprising at least one intercalator pseudonucleotide according to the present invention are used

to differentiate between target nucleic acid and/ or the target nucleic acid analogue and known types of single point mutations hereof.

5 Preferably extension of said oligonucleotide analogue indicates the presence of target nucleic acid and/ or target nucleic acid analogue. In a more preferred embodiment incorporated nucleotides are used to sequence target nucleic acid and/ or target nucleic acid analogue. In the most preferred embodiment, each type of labeled nucleotide is used to differentiate between target nucleic acid and/ or the target nucleic acid analogue and single point mutations hereof.

10

Preferably, targets are hybridized to solid support bound oligonucleotide analogues under high-stringency conditions. Then the solid support bound oligonucleotide analogues are extended with one labeled base at the 3'-end of the probe, which preferably is opposite the site of the expected mutation in the target nucleic acid or
15 nucleic acid analogue.

15

In a more preferred embodiment incorporated nucleotides are used to sequence target nucleic acid and/ or target nucleic acid analogue. In the most preferred embodiment, each type of nucleotide is used to differentiate between target nucleic
20 acid and/ or the target nucleic acid analogue and single point mutations hereof.

20

Alternatively differentiation between nucleic acids and/ or nucleic acid analogues and the mutants hereof can be carried out by ligation of labeled oligonucleotides or labeled oligonucleotide analogues.

25

In a preferred embodiment differentiation between target nucleic acids and/ or target nucleic acid analogues and the mutants hereof is carried out by the use of intermol[stavefej]ecular excimers, exciplexes, FRET complexes and/ or charge-transfer complexes. Hence it is a preferred embodiment to use labeled nucleic acids
30 and/ or nucleic acid analogues that will form intermolecular excimers, exciplexes, FRET complexes and/ or charge-transfer complexes, with the label of the nucleotide, nucleotide analogue, oligonucleotide or oligonucleotide analogue that are added to the 3' end of the oligonucleotide analogues comprising at least one intercalator pseudonucleotide according to the present invention. In an even more
35 preferred embodiment at least one of the labels is an intercalator pseudonucleotide.

35

Determining the presence or absence of hybridization

5 The presence or absence of a target sequence and/or a mutant sequence according to the invention may be determined by determining presence or absence of hybridization.

10 The determination of the extent of hybridization may be carried out by any of the methods well known in the art. If there is no detectable hybridization, the extent of hybridization is said to be 0. The oligonucleotide analogues according to the present invention comprise intercalator pseudonucleotides, which may be used to detect hybridization directly. In addition the oligonucleotide analogues according to the present invention may be coupled to one or more detectable labels.

15 The oligonucleotide analogues, which may be used as probes for detection, should be capable of specific interaction with target nucleic acids and/or nucleic acid analogues. When discriminating between target sequences and mutants sequences, the difference in melting temperature is a parameter that may be commonly used.

20 When using this strategy oligonucleotide analogues comprising intercalator pseudonucleotide provide a tool for the efficient discrimination. When at least one of the nucleotides in an oligonucleotide analogue comprising at least one intercalator pseudonucleotide is not hybridized the melting temperature of the hybrid will be lower than the melting temperature of a comparable hybrid wherein all nucleotides

25 are hybridized.

30 In particular, when all n nucleotides separating two intercalator pseudonucleotide of an oligonucleotide analogue are capable of hybridizing to the mutant sequence, but not capable of hybridising to the target sequence and the remaining nucleotides of the oligonucleotide analogue are capable of hybridising to the target sequence and the mutant sequence, a high melting temperature may be indicative of a mutation and a low melting temperature may be indicative of no mutation.

Vice versa, when all n nucleotides separating two intercalator pseudonucleotide of an oligonucleotide analogue are capable of hybridizing to the target sequence, but not all are capable of hybridising to the mutant sequence, and the remaining nucleotides of the oligonucleotide analogue are capable of hybridising to the target sequence and the mutant sequence, a high melting temperature may be indicative of no mutation and a low melting temperature may be indicative of mutation.

Corresponding target nucleic acids and/ or nucleic acid analogues or oligonucleotide analogues according to present invention may be labeled by any of several methods used to detect the presence of hybridized oligonucleotide analogues. The most common methods of detection are the use of ligands that bind to labeled antibodies, fluorophores or chemiluminescent agents. However, probes may also be labeled with ^3H , ^{125}I , ^{35}S , ^{14}C , ^{33}P or ^{32}P and subsequently detected by autoradiography. The choice of radioactive isotope depends on research preferences due to ease of synthesis, varying stability, and half-lives of the selected isotopes. Other labels include antibodies, which can serve as specific binding pair members for a labeled ligand. The choice of using the oligonucleotide analogues according to the present invention with or without one or more additional labeled nucleotides may depend on required sensitivity, the specificity as well as other factors. The choice label depends on the sensitivity, ease of conjugation with the probe, stability requirements, and available instrumentation.

Situations can be envisioned in which the detection probes comprises DNA or RNA. Such probes can be labeled in various ways depending on the choice of label. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes by several means such as by nick translation of double-stranded probes; by copying single-stranded M 13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive dNTP; by transcribing cDNA from RNA templates using reverse transcriptase in the presence of radioactive dNTP; by transcribing RNA from vectors containing SP6 promoters or T7 promoters using SP6 or T7 RNA polymerase in the presence of radioactive NTP; normal PCR including hot dNTPs; by tailing the 3' ends of probes with radioactive nucleotides using terminal transferase; or by phosphorylation of the 5' ends of probes using [^{32}P]-ATP and polynucleotide kinase.

Non-radioactive probes are often labeled by indirect means. Generally, one or more ligand molecule(s) is/are covalently bound to the probe. The ligand(s) then binds to an anti-ligand molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely.

Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

As mentioned the oligonucleotide analogues according to the present invention may in some embodiments also be conjugated directly to non-intercalator pseudonucleotide signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include, but is not limited to, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, AMPPD ([3-(2'-spiroamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxetane]) and 2,3-dihydrophthalazinediones, e.g., luminol.

The amount of labeled probe that is present in the hybridization medium or extraction solution may vary widely. Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA. By exploiting the high-affinity annealing properties of oligonucleotide analogues according to the invention towards certain nucleic acids, it may not be necessary to use substantial excesses of probe. Among the high-affinity oligonucleotide analogues, especially intercalator pseudonucleotide comprising oligonucleotide analogues are first choice candidates. Treatment with ultrasound by immersion of the reaction vessel into commercially available sonication baths can often accelerate the hybridization rates.

Typically, unlabelled species or excess label is removed before detection is carried out. Removal is often done by affixing either probe or target to a solid support (described herein above), where after washing can easily be done. After hybridization

at a temperature and time period appropriate for the particular hybridization solution used, the support to which the capturing probe (oligonucleotide analogue according to this invention):corresponding target DNA hybridization complex is attached is introduced into a wash solution typically containing similar reagents (e.g., sodium chloride, buffers, organic solvents and detergent), as provided in the hybridization solution. These reagents may be at similar concentrations as the hybridization medium, but often they are at lower concentrations when more stringent washing conditions are desired. The time period for which the support is maintained in the wash solutions may vary from minutes to several hours or more. Either the hybridization or the wash medium can be stringent. After appropriate stringent washing, the correct hybridization complex may now be detected in accordance with the nature of the label.

The probe may be conjugated directly with the label. For example where the label is fluorescent, the probe with associated hybridization complex substrate is detected by first irradiating with light of a particular wavelength. The sample absorbs this light and then emits light of a different wavelength, which is picked up by a detector (Physical Biochemistry, Freifelder, D., W. H. Freeman & Co. (1982), pp. 537-542). Where the label is radioactive, the sample is exposed to X-ray film or a phosphorimagescreen etc. Where the label is an enzyme, the sample is detected by incubation on an appropriate substrate for the enzyme. The signal generated may be a colored precipitate, a colored or fluorescent soluble material, or photons generated by bioluminescence or chemiluminescence.

When the label is an enzyme preferably the enzyme is capable of catalysing the production of a colored precipitate to indicate a positive reading, preferred enzymes according to the invention may be selected from the group consisting horseradish peroxidase, alkaline phosphatase, calf intestine alkaline phosphatase, glucose oxidase and beta-galactosidase. For example, alkaline phosphatase will dephosphorylate indoxyl phosphate, which will then participate in a reduction reaction to convert tetrazolium salts to highly colored and insoluble formazans.

Detection of a hybridization complex may require the binding of a signal-generating complex to a hybrid of corresponding target and oligonucleotide analogue. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-

conjugated probe and an anti-ligand conjugated with a signal. The binding of the signal generation complex is also readily amenable to accelerations by exposure to ultrasonic energy.

- 5 The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, attaching fluorescent or enzyme molecules or radioactive labels to the antibodies generates a signal (Tijssen, P. "Practice and Theory of Enzyme Immunoassays," Laboratory Techniques in Biochemistry and Molecular
10 Biology, Burdon, R. H., van Knippenberg, P.H., Eds., Elsevier (1985), pp. 9-20.)

Several kinds of fluorescence can be used for detection, including excimers, exiplexes, FRET complexes and charge-transfer complexes.

- 15 Specifically interesting assays are hybridization and fluorescence based detection assays involving single-base-extension of, or ligation to, oligonucleotide or oligonucleotide analogue probes affixed to a solid support. In these cases an affixed probe is hybridized to a corresponding target nucleic acid or corresponding target nucleic acid analogue, and the 3' end of the probe is extended by a DNA Polymerase or a
20 ligase. By this process labeled nucleotides, nucleotide analogues, oligonucleotides or oligonucleotide analogues are incorporated into the probe. Afterwards excess labels and optionally nucleic acid or nucleic analogue strands annealed to affixed probes are removed by stringent washes and remaining labels, now also affixed to the solid support through the extended probes, are detected. This method is well
25 suited for genotyping point mutations when choosing the different types of nucleotides used for extension to be labeled with different fluorescent labels. By choosing probe sequences to position the 3'-end of the probe next to the expected mutation in the corresponding target nucleic acid or corresponding target nucleic acid analogue, the signal of the particular fluorescent label will determine what nucleotide was in-
30 corporated and therefore also the nucleotide identity of this particular site in the target sequence. Instead of nucleotide extension, differently labeled oligonucleotides specifically hybridizing at the 3'-end of probes can also be used in ligation-mediated extension, to obtain similar information as mentioned above.

In the present context, the term "label" thus means a group that is detectable either by itself or as a part of a detection series. Examples of functional parts of reporter groups are biotin, digoxigenin, fluorescent groups (groups that are able to absorb electromagnetic radiation, e.g. light or X-rays, of a certain wavelength, and which subsequently reemits the energy absorbed as radiation of longer wavelength; illustrative examples are dansyl (5-dimethylamino)-1-naphthalenesulfonyl), DOXYL (N-oxy-4,4-dimethyloxazolidine), PROXYL (N-oxy-2,2,5,5-tetramethylpyrrolidine), TEMPO (N-oxy-2,2,6,6-tetra-methylpiperidine), dinitrophenyl, acridines, coumarins, Cy3 and Cy5 (trademarks for Biological Detection Systems, Inc.), erythrosine, coumaric acid, umbelliferone, Texas Red, rhodamine, tetramethyl rhodamine, Rox, 7-nitrobenzo-2-oxa-1-diazole (NBD), pyrene, fluorescein, Europium, Ruthenium, Samarium, and other rare earth metals, radioisotopic labels, chemiluminescence labels (labels that are detectable via the emission of light during a chemical reaction), spin labels (a free radical (e.g. substituted organic nitroxides) or other paramagnetic probes (e.g. Cu^{2+} , Mg^{2+}) bound to a biological molecule being detectable by the use of electron spin resonance spectroscopy), enzymes (such as peroxidases, alkaline phosphatases, β -galactosidases, and glucose oxidases), antigens, antibodies, haptens (groups which are able to combine with an antibody, but which cannot initiate an immune response by themselves, such as peptides and steroid hormones), carrier systems for cell membrane penetration such as: fatty acid residues, steroid moieties cholesteryl, vitamin A, vitamin D, vitamin E, folic acid peptides for specific receptors, groups for mediating endocytose, epidermal growth factor (EGF), bradykinin, and platelet derived growth factor (PDGF). Especially interesting examples are biotin, fluorescein, Texas Red, rhodamine, dinitrophenyl, digoxigenin, Ruthenium, Europium, Cy5, Cy3, etc.

Furthermore the term label in the present concept may also cover rather unspecific DNA stains. In assays where detection is based on the amount of double stranded nucleic acids or nucleic acid analogues only, unspecific DNA stains recognizing double stranded regions in a sequence independent manner are well suited. SYBR-green, Ethidium Bromide, DAPI and Acridine Orange are examples of widely used fluorescent stains for this purpose.

Determining the presence or absence of target sequences/mutant sequences by spectral properties of intercalator pseudonucleotides

- 5 The presence or absence of a mutation according to the present invention may be determined using a number of different assays. Preferably, the assays involve either determining melting temperature or determining spectral properties or a mixture of both.
- 10 Accordingly, in one embodiment of the present invention the presence or absence of the mutation is determined by determining the spectral properties of the oligonucleotide analogue comprising at least one intercalator pseudonucleotide after hybridization.
- 15 The spectral properties may be fluorescence properties, for example the spectral properties may be selected from the group consisting of monomer fluorescence excimer fluorescence, exciplex fluorescence, FRET and charge-transfer complex UV absorption band.
- 20 It is also possible to determine more than one spectral property, for example the spectral properties may be two or more selected from the group consisting of monomer fluorescence excimer fluorescence, exciplex fluorescence, FRET and charge transfer complex fluorescence, in particular the spectral properties may be monomer fluorescence and excimer or exciplex or FRET or charged transfer
- 25 fluorescence.

As discussed herein above, when intercalators in an oligonucleotide analogue are positioned in relation to each other so that they can form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex, then when

30 nucleobase pairs separating these two intercalators do base-pair that will preferably result in that said intercalators are not able to interact and hence form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex.

Accordingly, when an oligonucleotide analogue comprises two intercalator pseudonucleotides that are positioned in relation to each other so that they can form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex, low or essentially no high excimer fluorescence, exciplex fluorescence, FRET or charge-transfer complex_UV absorption band may be indicative of that all of the nucleotides in the region of n nucleotides separating the intercalators are hybridizing, and high excimer fluorescence, exciplex fluorescence, FRET or charge-transfer complex_UV absorption band may be indicative that at least one of the nucleotides in the region of n nucleotides separating the intercalators is unhybridized.

Preferably a pair of intercalator pseudonucleotides is positioned as next-nearest neighbors and only one mismatched base pair is present in the region surrounding the pair.

Furthermore, for some intercalators the monomer fluorescence changes upon hybridization of a double stranded nucleic acid. In particular, low monomer fluorescence of said intercalator might be indicative of no nucleotides in the region surrounding the intercalator pseudonucleotide are not hybridised, whereas high monomer fluorescence is indicative of that at least one of the nucleotides in the region surrounding the intercalator is not hybridised.

Preferably high monomer fluorescence is indicative of that one of the base pairs next to the intercalator pseudonucleotide is not hybridised.

Examples

The following examples illustrate selected embodiments of the invention and should not be regarded as limiting for the invention.

5

In the examples the following abbreviations are used:

ODN: Oligodeoxynucleotide

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INA: Intercalating nucleic acid corresponding to intercalator pseudonucleotide

Example 1

Preparation of an intercalator pseudonucleotide

15

1-Pyrenemethanol is commercially available, but it is also easily prepared from pyrene by Vilsmeier-Haack formylation followed by reduction with sodium borohydride and subsequent conversion of the alcohol with thionyl chloride affords 1-(chloromethyl)pyrene in 98% yield.

20

The acyclic amidite **5** (fig. 1) was prepared from (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol and 1-(chloromethyl)pyrene in 52% overall yield. The synthesis of **5** (fig. 1) is accomplished using KOH for the alkylation reaction, and using 80% aqueous acetic acid to give the diol **3** (fig. 1), which is protected with dimethoxytritylchloride (DMT-Cl) and finally reaction with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite affords target compound **5** (fig. 1) in 72% yield. 25

The yield in the latter reaction step was decreased from 72% to 14% when 2-cyanoethyl *N,N*-diisopropylchlorophosphor amidite was used as the phosphitylating reagent. The synthesis of the acyclic amidite **5** is shown schematically in figure 1.

30

1-Pyrenylcarbaldehyde

A mixture of *N*-formyl-*N*-methylaniline (68.0 g; 41.4 mL; 503 mmol) and *o*-dichlorobenzene (75 mL) is cooled on an ice bath and added phosphoroxychloride

156

(68g; 440 mmol) over 2 hours so that the temperature do not exceed 25 °C. Pulverized Pyrene (50 g; 247 mmol) is added in small portions over 30 min. and the reaction mixture is equipped with a condenser and heated at 90-95°C for 2 hours. After cooling to room temperature the dark red compound is filtered off and washed with benzene (50 mL.). Then it is transferred to water (250 mL) and stirred over night. The yellow aldehyde is filtered of and washed with water (3×50 mL). Recrystallized from 75% ethanol 3 times. Yeild: 30.0 g (52.7%).

10 1-Pyrenylmethanol

1-Pyrenylcarbaldehyde (10.0 g; 43.4 mmol) is dissolved in dry THF (50 mL) under inert atmosphere and NaBH₄ (0.82 g; 22 mmol) is added in small portions over 10 min. The reaction mixture is stirred over night at r.t. and crystallizing the product by pouring into stirring water (350 mL). The product is filtered off, washed with water (4×25 mL) and dried under reduced pressure. Recrystallized from ethyl acetate. Yeild: 8.54 g (84.7%).

20 1-(Chloromethyl)-pyrene

1-Pyrenylmethanol (6.40 g; 27.6 mmol) is dissolved in a mixture of pyridine (3.3 mL; 41.3 mmol) and CH₂Cl₂ (100 mL) and the mixture is cooled to 0°C. SOCl₂ (3.0 mL; 41.3 mmol) is added slowly over 15 min. and the temperature is allowed to rise slowly to r.t. Stir over night. The mixture is poured into stirring water (200 mL) and added CH₂Cl₂ (100 mL). The mixture is stirred for 30 min. The organic phase is washed with 5% NaH₂CO₃ (2×75 mL) and brine (2×75 mL) respectively, dried with sodium sulfate and concentrated under reduced pressure. Recrystallized from toluene/ petroleum ether. Yield 6.75 g (97.8%).

30 (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol

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Pulverized KOH (25 g) and 1-(Chloromethyl)-pyrene (6.0 g; 23.9 mmol) is added to a solution of (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (2.6 g; 19.7 mmol) in dry toluene (250 mL). The mixture is refluxed under Dean-Stark conditions in 16h, then cooled to r.t. and added water (150 mL). The organic phase is washed with water (3×100 mL) dried with a combination of magnesium sulfate and sodium sulfate and concentrated under reduced pressure to a thick oil. Silica gel chromatography (CH₂Cl₂) afforded the pure compound in 6.1 g (90%).

10 (R)-3-(1-Pyrenemethoxy)-propane-1,2-diol

(S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (6.1 g; 17.6 mmol) is added to a mixture of acetic acid and water (100 mL; 4:1) and is stirred at r.t. for 19h. Concentrated under reduced pressure. Giving an oil in quantitatively yield.

15

(S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol

(R)-3-(1-Pyrenemethoxy)-propane-1,2-diol (760 mg; 2.48 mmol) is dissolved in dry pyridine (20 mL) and added dimethoxytrityl chloride (920 mg; 2.72 mmol). The reaction mixture is stirred in 24h and concentrated under reduced pressure. Purified by silica gel chromatography (ethyl acetate/ cyclohexane/ triethylamine 49:49:2) to give a white foam. Yield 1.20 g (79.5%).

25

Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol

(S)-1-(4,4'-Dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol (458 mg; 753 μmol), 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphan (453 mg; 429 μL; 1.51 mmol) and diisopropylammonium tetrazolide (193 mg; 1.13 mmol) is mixed in dry CH₂Cl₂ (7 ml) and stirred under nitrogen atmosphere for 6 days. Purified by silica gel

30

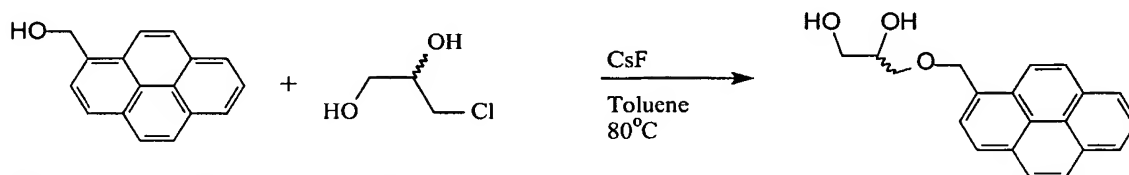
158

chromatography (ethyl acetate/ cyclohexane/ triethylamine 49:49:2) and dried under reduced pressure. Yield 438 mg (72%) as a white foam.

Example 2

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Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol



Scheme 1: Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol

10

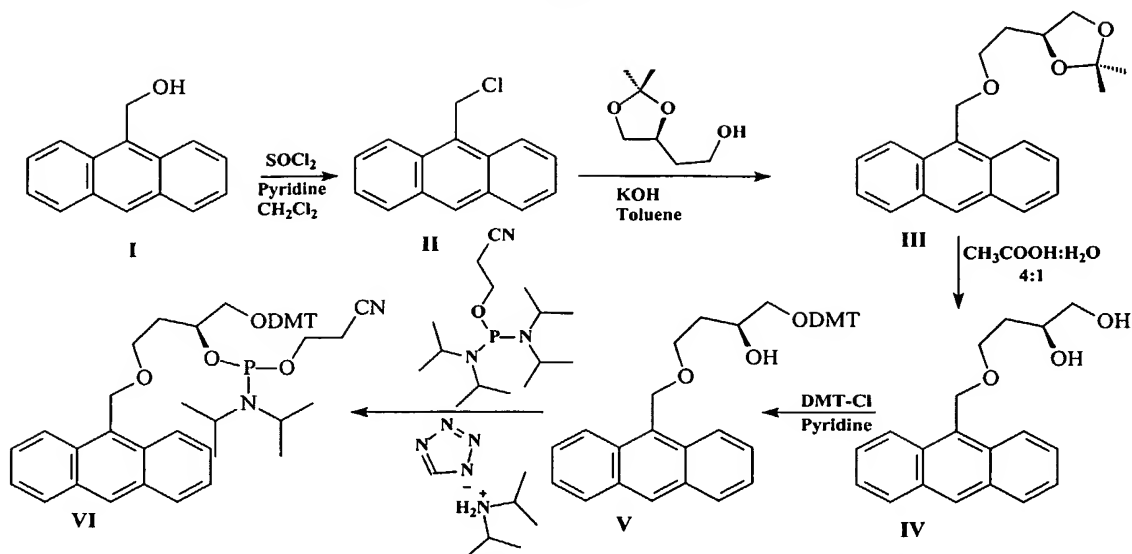
1-Pyrenylmethanol (232 mg; 1.0 mmol) is dissolved in hot toluene (2 mL over Na). CsF (7 mg; 0,046 mmol) is added and stirred for approx. 1h at room temperature when 3-chloro-1,2-propanediol (170 mg; 1.53 mmol) is added. The mixture is stirred at 80°C for 2h, cooled off to room temperature and the precipitated product is separated from the mixture by filtration. Washed with cold toluene (2 × 1 mL). Yield 220 mg (72%).

15

Example 3

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Synthesis of the 2-O phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylnmethyl)-1,2,4-butanetriol



Scheme 2: Schematic view of the synthesis of the 2-O-phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol

5 9-anthracenemethylchlorid (II)

9-anthracenemethanol (0.81 g; 3.89 mmol; I) was dissolved in dry pyridine (467 μ L; 5.83 mmol) and dry CH_2Cl_2 . Under stirring and at 0°C SOCl_2 (423 μ L; 5.83 mmol) was added dropwise, and the mixture was stirred for 24h during which the temperature is allowed to rise to r.t. within 2h. The reaction is poured onto stirring H_2O (60 mL) and was added additional CH_2Cl_2 (40 mL). The organic phase was washed with a 5% NaHCO_3 (100 mL) solution, brine (100 mL) and water (100 mL) respectively. Dried over Na_2SO_4 and concentrated *in vacuo*. Yield 665 mg (75%).

15 1,2-D-□□-isopropylidene-4-(9-anthracenylmethyl)-1,2,4-butanetriol (III)

9-anthracenemethylchlorid (628 mg; 277 mmol) was dissolved in dry toluene (25 mL over Na) and 2-[(S)-2',2'-dimethyl-1',3'-dioxalan-4'-yl]-ethanol (506 mg; 3.5 mmol) and 3 small spoons of KOH was added. The mixture was connected to a Dean-Stark apparatus and stirred under reflux conditions over night. The reaction mixture was slowly cooled to r.t. and washed with H_2O (4 \times 25 mL). Dried over Na_2SO_4 and concentrated *in vacuo*.

160

4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (IV)

To the dried compound was added 80% AcOH (50 mL) and the reaction mixture was stirred in 24h at r.t. The mixture was concentrated in vacuo over night and purified by short, fast silica gel chromatography (impurities was first eluted with CH₂Cl₂, and product was then eluted with 5% MeOH in CH₂Cl₂). Yield 56.3% over 2 steps.

1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (V)

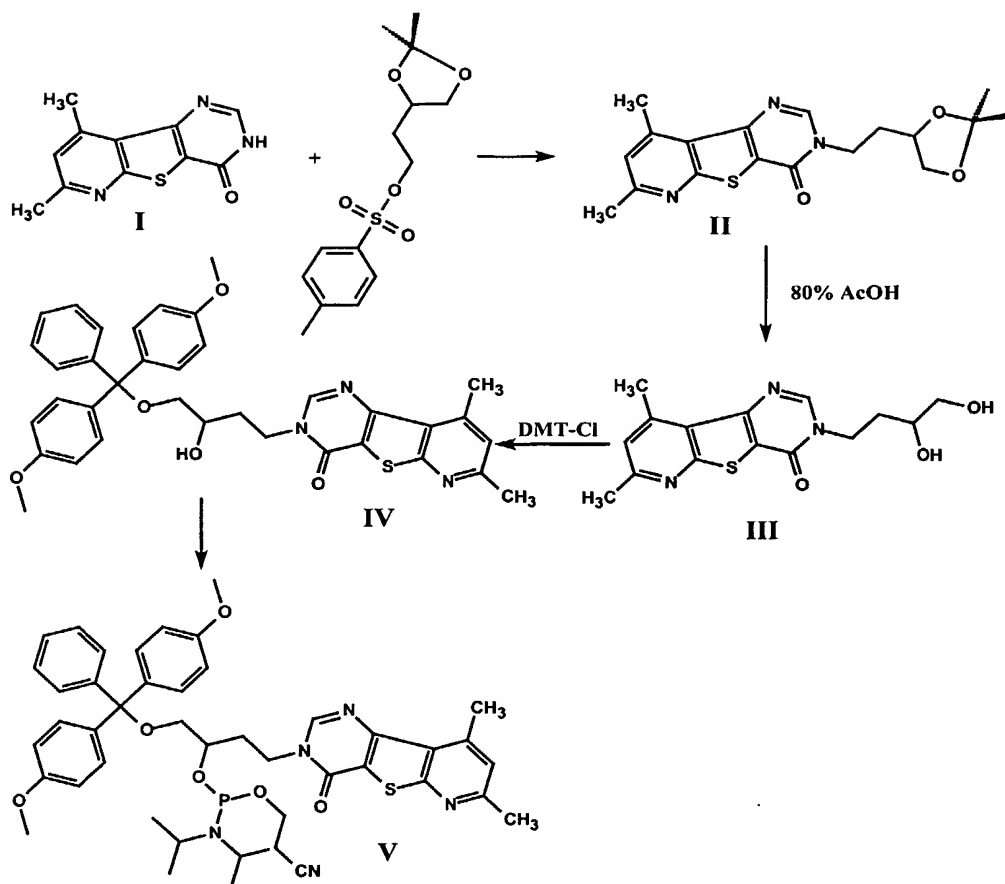
The diol (425 mg; 1.40 mmol) and DMT-Cl is mixed in dry pyridine (5 mL) and stirred at r.t. for 36h. The reaction mixture was concentrated in vacuo and purified by silica gel chromatography (EtOAc:°C₆H₁₂:N(Et)₃ 63:35:2). Co-evaporated with ether (5 mL over Na) after which a yellowish foam was isolated. Yield 630 mg (74%).

The phosphoramidite of 1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (VI)

The DMT protected anthracene compound was dissolved in dry CH₂Cl₂ (7 mL) and diisopropylammonium tetrazolide (252 mg; 1.5 mmol) and 2-Cyanoethyl N,N,N',N'-tetraisopropyl Phosphane was added. The reaction mixture was stirred for 20h at r.t. Concentrated in vacuo and purified by silica gel chromatography (EtOAc:°C₆H₁₂:N(Et)₃ 24:74:2). Co-evaporated with ether (5 mL over Na) to give a yellowish foam (67%).

Example 4

Synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)



Scheme 3: Synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (I)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one was prepared according to literature procedures.^{1,2,3}

3-N-((S)-2'',2''-diethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (II)

¹ Schimdt, U. & Kubitzek, H., Chem. Ber., 93, 1559 (1960)

² Hassan, K. M. et al. Phosphorous, Sulfur, Silicon Relat. Elem., 47, 181 (1990)

³ Gewald, K. & Jansch, H. J., Prakt. Chemie 313-320 (1976)

162

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (1.16 g; 5.0 mmol) was suspended in anhydrous DMF (20 mL) and NaH (0.2 g; 5.0 mmol, 60% dispersion in mineral oil) was added. The mixture was stirred for 2h until all H₂ evolving ceased. Then (S)-2,2-dimethyl-1,3-dioxalane-4-ethanoyl-O-para-toluenesulfonate (0.78 g; 5.1 mmol) was added in one portion and the mixture was stirred for 24h at 80°C. The mixture was evaporated to dryness *in vacuo*, co evaporated with dry toluene (3 × 10 mL) *in vacuo* and the residue was purified silica gel chromatography (5% EtOAc in CHCl₃) to get a colorless product. Yield 0.81 g; 45%.

10 (S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (III)

3-N-((S)-2'',2''-dimethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (0.75 g; 2.1 mmol) was stirred at r.t. in 80% AcOH (20 mL) for 24h. The product was obtained by concentration *in vacuo* and co-evaporation with EtOH. Purified by silica gel chromatography (5% MeOH in CHCl₃) to get the colorless product. Yield 0.5 g (75%).

20 (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (IV)

(S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (0.6 g; 1.9 mmol) was dissolved in dry pyridine (5 mL) and DMT-Cl (0.71 g; 2.1 mmol) was added. Stirred at r.t. over night. Concentrated *in vacuo* and co evaporated using dry toluene (3 × 10 mL). The residue was purified by silica gel chromatography (EtOAc: C₆H₁₂:N(Et)₃ 49:49:2) to yield a white foam. Yield 0.77 g (65%)

30 Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

(S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (310 mg; 0.5 mmol) was dissolved under nitrogen in anhydrous dichloromethane (10 mL). Diisopropylammoniumtetrazolide (0.11 g; 0.67 mmol) was added followed by dropwise addition of 2-Cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.3 g; 1.0 mmol) the reaction was stirred over night

163

under nitrogen atmosphere, concentrated *in vacuo* and purified by silica gel chromatography (EtOAc:^cC₆H₁₂:N(Et)₃ 49:45:12) to give a white foam. Yield 345 mg (84%)

5 Example 5

Duplexes with dangling ends.

10 To investigate the stacking ability of the nucleoside analogue 5 (fig. 1) it was incorporated into the 5' end of two different self-complementary strands (5'-XCGCGCG and 5'-XTCGCGCGA).

15 The ODN synthesis is carried out on a Pharmacia LKB Gene Assembler Special using Gene Assembler Special software version 1.53. The pyrene amidite is dissolved in dry acetonitrile, making a 0.1M solution and inserted in the growing oligonucleotides chain using same conditions as for normal nucleotide couplings (2 min. coupling). The coupling efficiency of the modified nucleotides is greater than 99%. The ODNs are synthesized with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-20 1 column. Buffer A: 950 ml. 0.1 M NH₄⁺HCO₃⁻ + 50 ml MeCN pH =9.0; buffer B: 250 ml. 0.1 M NH₄⁺HCO₃⁻ + 750 ml MeCN pH = 9.0. Gradients: 5 min. 100% A, linear gradient to 100% B in 40 min., 5 min. with 100% B, linear gradient to 100% A in 1 min. and the 100% A in 29 min (product peak ≈ 37 min.). The ODNs were DMT deprotected in 925 µl H₂O + 75 µl CH₃COOH and purified by HPLC again using the 25 same column, buffer system and gradients (product peak ≈ 26 min.). To get rid of the liable salts, the ODNs were re-dissolved in 1 ml of water and concentrated *in vacuo* three times.

30 All oligonucleotides were confirmed by MALDI-TOF analysis made on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and PECSS software package ver. 4.3. Melting temperature measurements of the self-complementary sequences are made in 1 M NaCl, 10 mM Na•Phosphate pH 7.0, 35 1.5 µM of each DNA strand. All other ODNs are measured in a 150 mM NaCl, 10

164

mM, Na•Phosphate, 1 mM EDTA pH 7.0, 1.5 μ M of each strand. All melting temperatures giving are with an uncertainty on ± 0.5 °C.

5 The Amber forcefield calculations were done in MacroModel 6.0 and 7.0 with water as solvent and minimization is done by Conjugant Gradient method. The starting oligonucleotide sequences for calculation with the inserted pyrenes is taken from Brookhavens Protein Databank, and modified in MacroModel before minimazation is started. Lam and Au-Yeung solved a structure of a self-complementary sequence, equal to the one used in this work, by NMR. Their structure is prolonged with the
10 pyrene amidite at the 5'-end of each strand and used for the structural calculations. The other sequence is a 13-mer highly conserved HIV-1 long terminal repeat region. G-7 is replaced by the pyrene amidite and calculations are made with and without an across lying C-nucleotide. The pyrene is placed in the interior of the duplex from the beginning. All bonds are free to move and to rotate.

15

The melting temperature of modified and unmodified, self-complementary DNA are shown in Figure 33. Incorporation of the pyrene amidite in the 5' end as a dangling end stabilises the DNA duplex with 19.2 °C – 21.8 °C (8.6 °C – 10.9 °C per modifi-
20 cation) depending on the underlying base pair. The stabilizations of the duplexes due to incorporation of 5 at the 5' termini of the nucleic acid strands are similar to those found by Guckian et al. who inserted a pyrene nucleoside at the 5' termini of self complementary ODNs (oligo deoxynucleic acids). The stabilisation can be explained by calculations using "MacroModel" which predict a structure were the pyrene moiety interacts with both nucleosides in the underlying basepair (figure 2).

25

165

Table 1. Melting temperatures of self-complementary sequences with 5' modification..

| | | T_m (°C) | ΔT (°C) |
|----|-------------------|------------|-----------------|
| 5' | C-G-C-G-C-G | 41.0 | |
| 3' | G-C-G-C-G-C | | |
| 5' | T-C-G-C-G-C-G-A | 46.9 | |
| 3' | A-G-C-G-C-G-C-T | | |
| 5' | X-C-G-C-G-C-G | 62.8 | 21.8 |
| 3' | G-C-G-C-G-C-X | | |
| 5' | X-T-C-G-C-G-C-G-A | 64.1 | 17.2 |
| 3' | A-G-C-G-C-G-C-T-X | | |

5 Example 6

End-positioned intercalating pseudonucleotides - stabilisation dependent on intercalator-linker length

10 **Introduction**

In this example is shown the dependence on linker length for the increase of affinity by the addition of intercalating pseudonucleotide to the 5'-end of an oligonucleotide. There is further more shown two examples of intercalating pseudonucleotides of comparable stabilisation effect.

15

Material and Methods

Oligonucleotides:

20

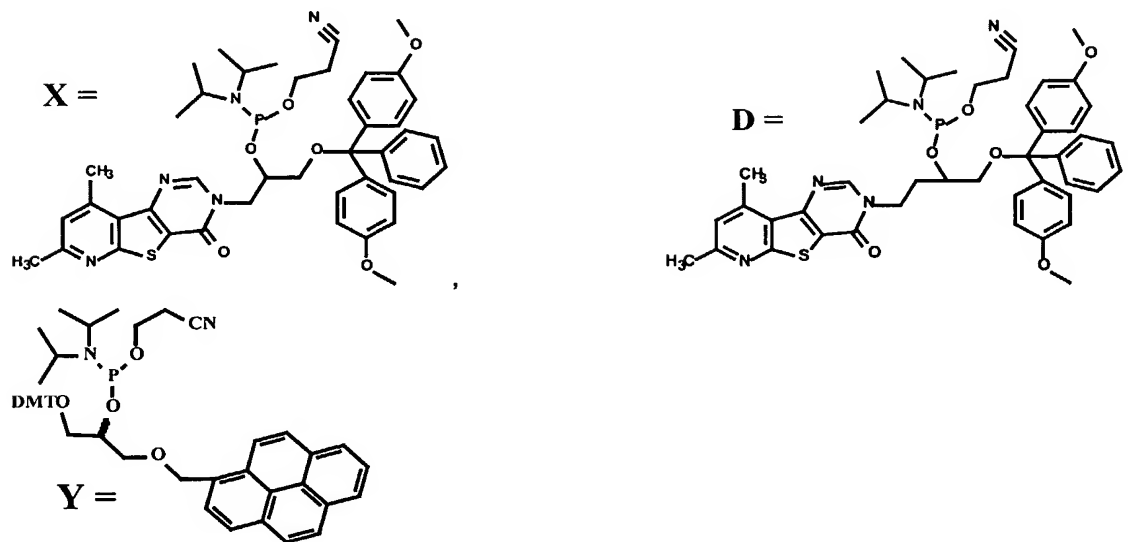
Probe I: 3'-CGA ACT CX
 Probe II: 3'-CGA ACT CD
 Probe III: 3'-CGA ACT CY
 Ref: 3'-CGA ACT C

166

Target: 5'-GCT TGAG

Below is shown the amidites that were used in the preparation of the above mentioned oligonucleotides:

5



10

All hybridisation experiments were carried out with 1.5 μ M of both target and probe strands in 2 mL of a buffer solution containing:

140mM NaCl

10 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$

1 mM EDTA

15

pH = 7.0

20

The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of $\pm 1.0^\circ\text{C}$ as determined by repetitive experiments.

Results and Discussion

The results of the melting experiments is shown in Table 5:

| Name | Melting temperature (°C) | ΔT_m (°C) |
|-------------------------|--------------------------|-------------------|
| Hybridisation to Target | | |
| Ref | 22.8 | ---- |
| Probe I | 28.4 | 5.6 |
| Probe II | 34.4 | 11.6 |
| Probe III | 33.8 | 11.0 |

5

The difference in melting temperature between probe I and II is due to the short linker of probe I. Hence it is important that the combined length of linker and intercalator is optimal, to obtain a large increase in affinity between intercalating pseudonucleotide modified oligonucleotides and their target DNA sequences. Probes II and III have nearly the same affinity for their target sequences, even though the intercalating moieties in the two probes are very different. This shows that the intercalating pseudonucleotides are a class of compounds that, dependent on the wanted feature it should introduce into an oligonucleotide or oligonucleotide analogue, it should be designed by more or less strict rules.

15

Example 7

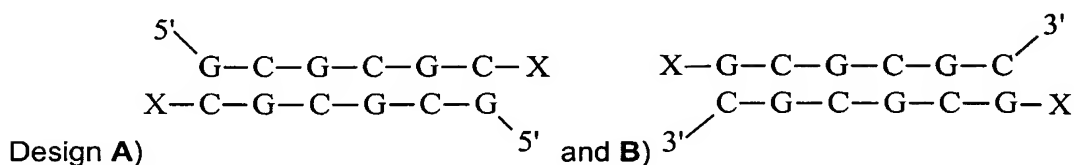
Oligonucleotides synthesized on universal supports – obtaining 3' intercalator pseudonucleotide modified oligonucleotides

In a preferred embodiment of the present invention, oligonucleotides or oligonucleotide analogues comprise intercalating pseudonucleotides at either or both ends. In this example it is shown that oligonucleotides or oligonucleotide analogues with intercalating pseudonucleotides in the 3'-end can be synthesised using Universal supports. It is furthermore shown that selfcomplementary oligonucleotides comprising intercalating pseudonucleotides positioned in the 3'-end form very thermal stable hybrids.

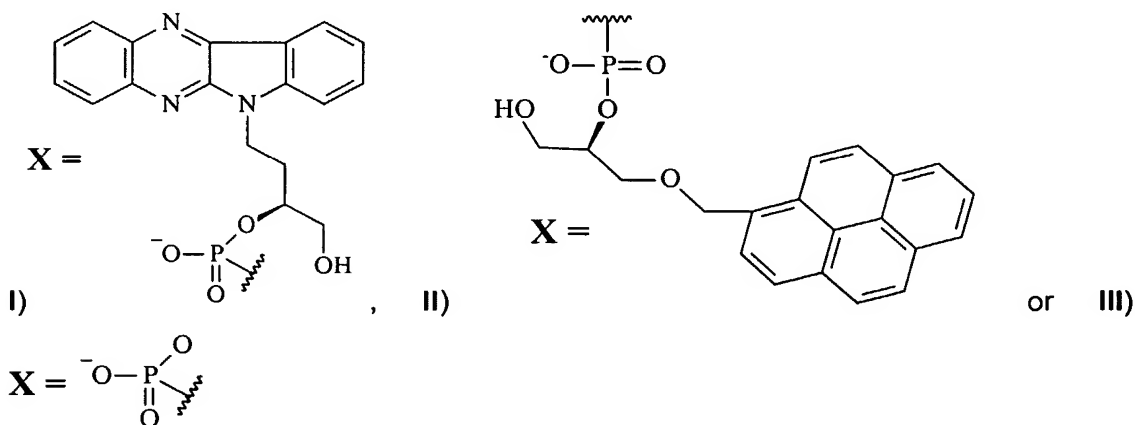
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Material and methods

The following two types of self-complementary probes were synthesised. Design A was synthesized using a universal support while B was synthesized using standard nucleotide coupled columns and procedures:



Two different intercalating pseudonucleotides were used for design A (I and II), and II was used for design B as well. One reference sequence without any intercalating pseudonucleotides (III) was synthesised. Hence X represents either:



After synthesis the oligo nucleotide analogues were treated with 2% LiCl in a 32% NH₄OH solution in order to remove protection groups from the heterocyclic amines and to cleave the oligonucleotide from the universal support. Oligonucleotides comprising intercalating pseudonucleotides were tested on MALDI-TOF and found at the expected values.

All hybridisation experiments were carried out with 1.5 μM of both target and probe strands in 1 mL of a buffer solution containing:

140mM NaCl

10 mM Na₂HPO₄•2 H₂O

1 mM EDTA

5 The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of $\pm 1.0^\circ\text{C}$ as determined by repetitive experiments.

10

Results and discussion

| <u>X\ Design</u> | A | B |
|------------------|----------|----------|
| I | 57.3°C | ----- |
| II | 59.2°C | 62.8°C |
| III | 41.0°C | 41.0°C |

15 From the above table it can be seen that the insertion of intercalating pseudonucleotides in either end of an oligonucleotide increases the affinity for a complementary target nucleic acid. It is also shown that intercalating pseudonucleotides can be inserted into the 3' end of an oligonucleotide or oligonucleotide analogue by using standard universal base chemistry.

20 Example 8

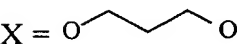
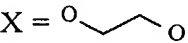
Substituting a nucleotide with a 1-O-(pyrenylmethyl)glycerol nucleotide.

25 The ODN synthesis is carried out as described in example 5. Phosphoramidite 5 (fig. 1) is prepared as described in example 1.

UV melting temperature measurements (Table 2) where a G nucleotide is replaced by the flexible, abasic linkers ethylene glycol and 1,3-propandiol shows, not surpris-

ingly, a decrease in duplex stability compared to the unmodified, fully complementary sequence. The required DMT protected cyanoethyl N,N-diisopropylphosphoramidites of ethylene glycol and 1,3-propandiol were synthesized by standard methods. Having the 1-O-(pyrenylmethyl)glycerol nucleotide in the same position instead
5 of the abasic diols increases the melting temperature by 16.4 °C – 18.0 °C for the DNA/DNA duplex, indicating that the pyrene is co-axial stacking with both sides of the duplex, as the stabilization per modification exceeds the effect of placing the pyrene module at one end of a duplex (Table 1). Calculations from "MacroModel" shows that the pyrene module only makes a minor distortion of the double helix
10 when intercalated into the duplex, having interaction with nucleobases both to the 5' side and to the 3' side of the intercalation site (Figure 3). The stabilization of the duplex by co-axial stacking of the pyrene moiety is not large enough to compensate for the loss in binding affinity due to the reduced number of hydrogen bonds by substitution of G with the pyrene moiety, the modified duplex being less stable than the
15 unmodified fully complementary by 8.6 °C. The same trend is found for DNA/RNA duplexes although these have lower melting temperatures in general than the corresponding DNA/DNA duplexes. The stabilization of the pyrene moiety is only 8.2 °C for the DNA/RNA duplex when compared with ethylene glycol whereas the stabilization is 16.4 for the DNA/DNA duplex. The pyrene insertion results in an improved
20 discrimination between ssDNA and ssRNA with 9.0 °C difference in the melting temperatures of their corresponding duplexes.

Table 2 DNA/DNA and DNA/RNA duplexes where one nucleotides is either an abasic, flexible linker, the pyrene module (5), a deletion or a complementary G.

| | | 171 | | |
|-------|---|------------------------------|----------------------------|---|
| | | U—U/ U—U/ | | |
| | | 5' A—G—C—T—T—G—C—C—T—T—G—A—G | | |
| | | 3' T—C—G—A—A—C—X—G—A—A—C—T—C | | |
| Entry | | DNA T _m (°C) | RNA T _m (°C) | Discrimination ΔT _{m, DNA-RNA} (°C) |
| 1 | X =  | 26.0 | 25.8 | 0.2 |
| 2 | X =  | 27.6 | 26.8 | 0.8 |
| 3 | X = 5 | 44.0 | 35.0 | 9.0 |
| 4 | X = - (12-mer) | 35.2 | 29.6 | 4.6 |
| 5 | X = G (13-mer) | 52.6 | 47.2 | 5.4 |

Example 9

5 1-O-(pyrenylmethyl)glycerol as a bulge.

Normally the introduction of a bulge into the double helix decreases the melting temperature. This is also observed here (Table 3), but if the pyrene module is built in as the bulge, the melting temperature of the DNA duplex goes up by 3°C. This is in accordance with the observations made by Ossipov et al., finding it necessary to introduce a bulge to prevent a large destabilization of the duplex when introducing a non-Watson-Crick binding intercalator. One pyrene moiety stabilises the duplex by 11.2 °C compared to the flexible ethylene glycol linker indicating that the pyrene moiety is intercalated into the duplex.

The difference in the melting temperature between the pyrene modified DNA/DNA duplex and the pyrene modified DNA/RNA duplex is increased to 12.6 °C when inserting one pyrene modification as a bulge. This difference is 7.4 °C larger than in the unmodified duplexes and much larger than the differences between the duplexes containing natural nucleoside or flexible ethylene glycol bulges. This means that the pyrene moiety is selective and only able to stabilize DNA/DNA duplexes and not the DNA/RNA duplex. For the latter duplex it occurs that the duplex have the

same melting temperature with the glycerol linker than with the pyrene moiety, indicating that the pyrene does not intercalate into the strands.

5 Structural calculation of the pyrene modified DNA/DNA structure (figure 4) shows that the pyrene module only makes minor distortion in the duplex, and that the linker introduces enough flexibility in the backbone to have a distance of 3.4 Å between the pyrene moiety and the nucleobases of the same strand. The nucleobases of the opposite strand has a little shorter spacing between the pyrene moiety and the nucleobases than the optimum 3.4 Å.

10

To investigate the discrimination and stabilization phenomena further, some ODNs with two insertions of the pyrene amidite were prepared, to see if the effects of the pyrene module is additive, and indeed it is. The results (Table 3) show that, depending on the distance between two insertions and their neighboring base pairs, it is possible to stabilize the double inserted pyrene DNA/DNA duplex up to 13.4 °C (6.7 °C per modification) compared to the natural DNA duplex. The destabilization of the pyrene modified DNA/RNA duplex is also somewhat additive, so that the difference in melting temperature between the pyrene modified DNA/DNA duplex and the pyrene modified DNA/RNA duplex is up to 25.8 °C when two pyrene modifications is inserted. For both stabilization and discrimination the best results are obtained, when the insertions are separated by four base pairs. When two insertions of the pyrene amidites are placed next to each other in the ODN there is a decrease in melting temperature of 5.2 °C compared to the unmodified DNA/DNA duplex and a decrease of 8.2 °C compared to the mono modified duplex. It is noteworthy that one basepair between two insertions in the DNA/DNA duplex is sufficient to improve DNA/DNA stabilization and DNA/RNA discrimination, when compared with the duplex with only one insertion.

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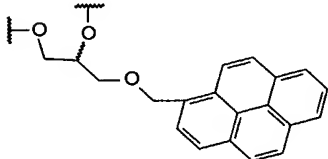

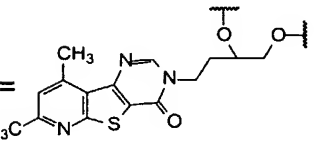

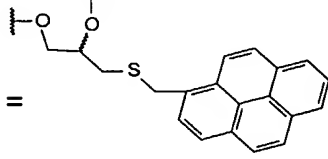

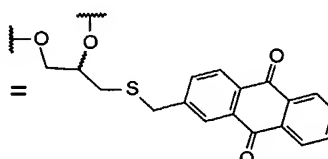

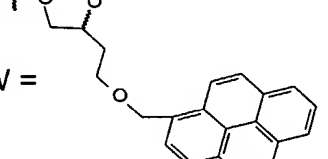

Table 3 Melting temperatures of oligonucleotides with different substitutions hybridized to either DNA or RNA.

Example 10**Five different intercalating pseudonucleotides as bulge insertion with increased affinity for the complementary DNA target**

5

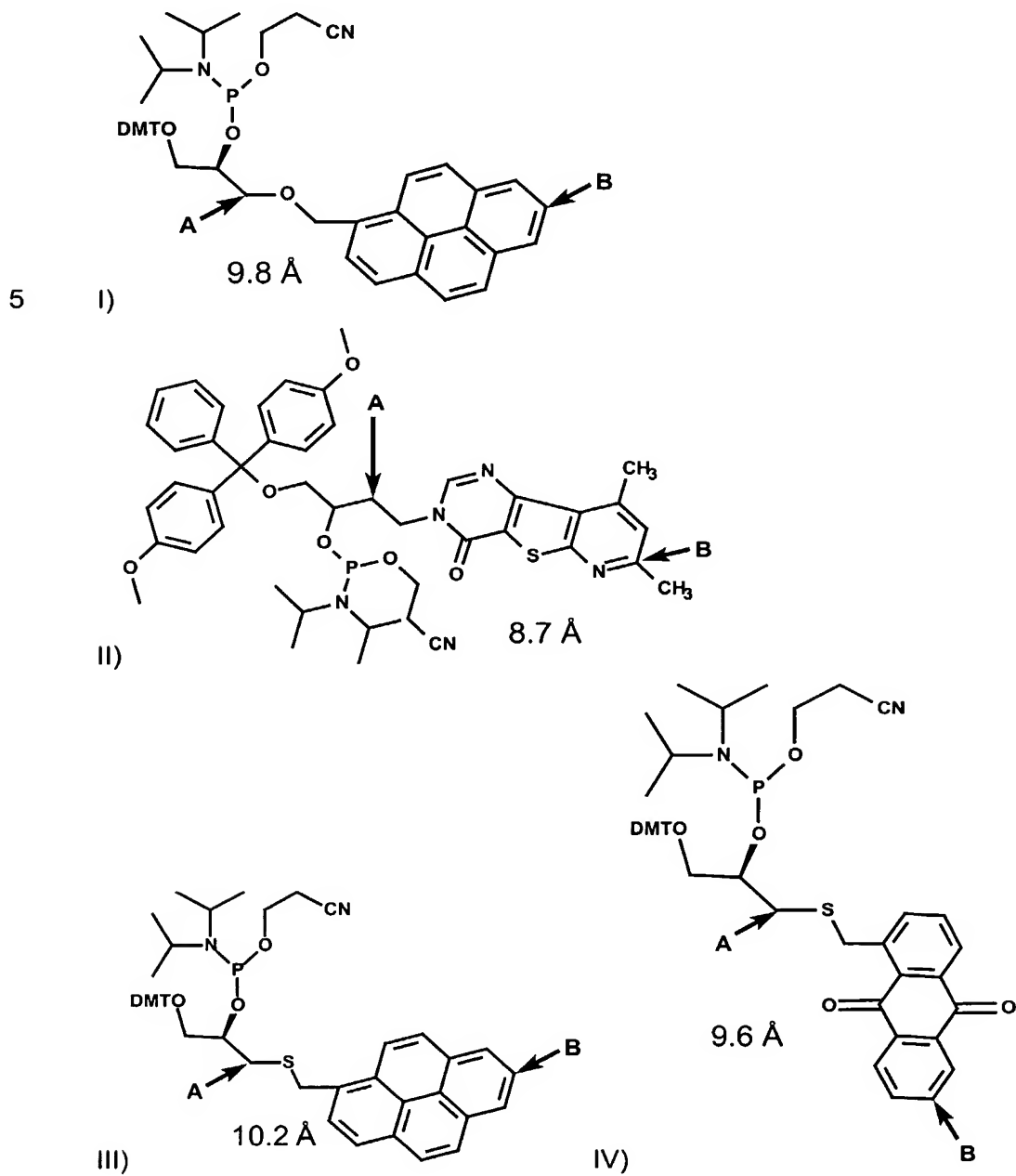
Below is shown an overview of five different intercalating pseudonucleotides inserted in the middle of a DNA oligonucleotide. When hybridised to target said intercalating pseudonucleotides act as bulge insertions. All intercalating pseudonucleotide modified oligonucleotides shown here have an increased affinity for the complementary DNA target compared to the unmodified oligonucleotide:

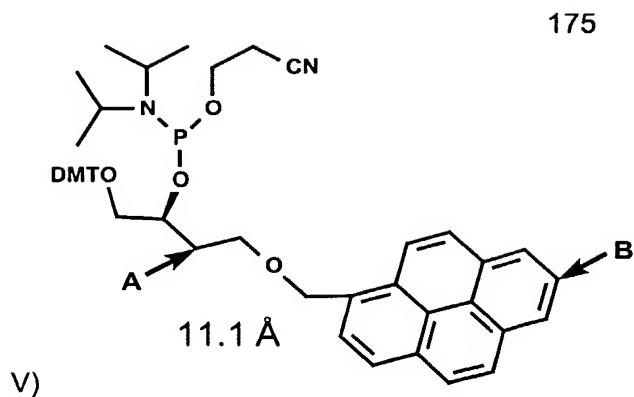
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| Target DNA strand | 5' A-G-C-T-T-G-C-T-T-G-A-G | T _m , DNA |
|---|---|----------------------|
| | 3' T-C-G-A-A-C-G-A-A-C-T-C | 47.4 °C |
|  | 3' T-C-G-A-A-C  G-A-A-C-T-C | 50.4 °C |
|  | 3' T-C-G-A-A-C  G-A-A-C-T-C | 50.6 °C |
|  | 3' T-C-G-A-A-C  G-A-A-C-T-C | 49.4 °C |
|  | 3' T-C-G-A-A-C  G-A-A-C-T-C | 49.2 °C |
|  | 3' T-C-G-A-A-C  G-A-A-C-T-C | 48.8 °C |

174

Below is shown the combined length of the linker and intercalator, it is clear that all of the shown examples have nearly the same combined length of intercalator and linker (9.9 ± 1.3 Å).



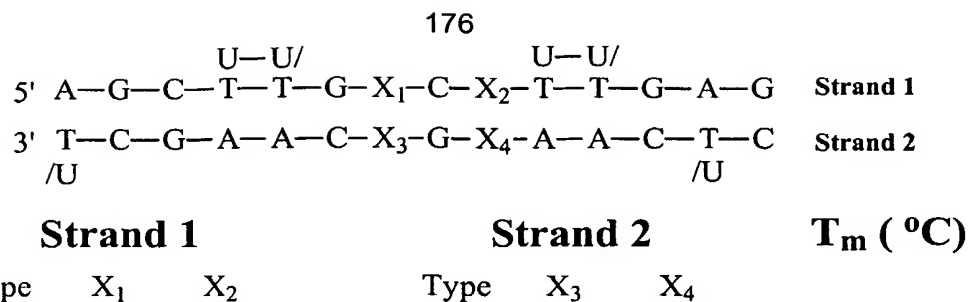


We can from this conclude that intercalating pseudonucleotides are a broad group of compounds that obeys some simple rules regarding the combined length of the intercalator and linker.

Example 11

Higher affinity for DNA - lower affinity for RNA.

In this example it is shown that when the target nucleic acid sequence for a probe is DNA, the melting temperature of the hybrid increases by introduction of intercalating pseudonucleotides into the probe – regardless if the probe is DNA or RNA (see Table 4 below). Additionally the affinity for a RNA target is reduced regardless if the probe is DNA or RNA. Hence, intercalator pseudonucleotides can be introduced to oligonucleotides or oligonucleotide analogues giving the oligonucleotide or oligonucleotide analogue increased affinity for DNA and reduced affinity for RNA and RNA-like compounds like LNA, 2'-O-METHYL RNA .



DNA duplexes and hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| DNA | - | - | DNA | - | - | 48.4 |
| DNA | 5 | 5 | DNA | - | - | 51.4 |
| DNA | - | - | DNA | 5 | 5 | 48.4 |

RNA duplexes and hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| RNA | - | - | RNA | - | - | 57.8 |
| RNA | 5 | 5 | RNA | - | - | 46.8 |
| RNA | - | - | RNA | 5 | 5 | 47.2 |

DNA/RNA hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| DNA | - | - | RNA | - | - | 42.2 |
| DNA | 5 | 5 | RNA | - | - | 34.2 |
| DNA | - | - | RNA | 5 | 5 | 45.2 |

5 **Table 4: Three different situations. At the top: DNA duplex affinity is increased or unaltered by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. In the middle: The RNA duplex is destabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. At the bottom: Here it is shown how the hybrid between a DNA and a RNA strand is stabilized by intercalator pseudonucleotides if these are comprised by the**
 10 **RNA strand. Furthermore it is shown than when incorporated into the DNA strand the affinity for RNA is decreased. 5 = amidite 5 from example 1 incorporated into the strand according to the procedure described herein above.**

By different relative positioning of the intercalator pseudonucleotides it is possible to gain higher affinity for DNA than shown in the Table above. Examples of this are given in the table in example 9.

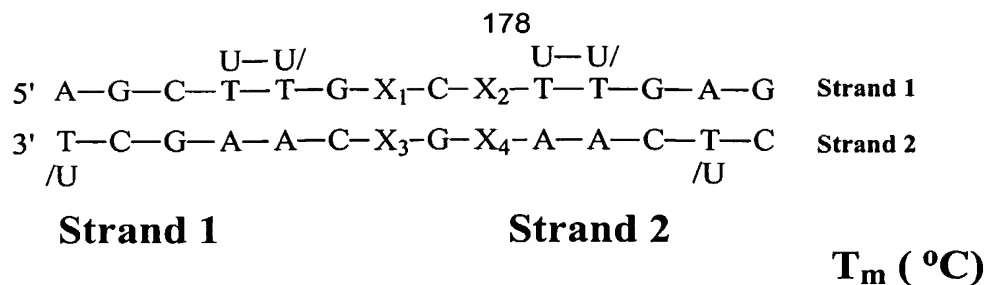
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Example 12

Reduced cross-hybridization

- 10 Cross-hybridization between two corresponding oligonucleotides comprising at least one intercalator pseudonucleotide with reduced affinity depending of the relative positioning of the intercalator pseudonucleotides. In the table below it is shown how the melting temperature is decreased if intercalator pseudonucleotides are positioned right opposite each other.

15



DNA duplexes and hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| DNA | - | - | DNA | - | - | 48.4 |
| DNA | 5 | 5 | DNA | - | - | 51.4 |
| DNA | 5 | 5 | DNA | 5 | 5 | 42.6 |

RNA duplexes and hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| RNA | - | - | RNA | - | - | 57.8 |
| RNA | 5 | 5 | RNA | - | - | 46.8 |
| RNA | 5 | 5 | RNA | 5 | 5 | 45.4 |

RNA and DNA hybrids

| | | | | | | |
|-----|---|---|-----|---|---|------|
| DNA | - | - | RNA | - | - | 42.2 |
| DNA | - | - | RNA | 5 | 5 | 45.2 |
| DNA | 5 | 5 | RNA | 5 | 5 | 37.8 |

Table 5: Three different situations. At the top: DNA duplex is stabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. If intercalator pseudonucleotides are positioned in relation to each other, so that they are in close vicinity of each other when the oligonucleotides or oligonucleotide analogues are hybridized the melting temperature is decreased compared to when only one strand comprises intercalator pseudonucleotides. In the middle: RNA duplex is destabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strand comprise the intercalator pseudonucleotides. At the bottom: Here it is shown how the hybrid between a DNA and a RNA strand is stabilized by intercalator pseudonucleotides if these are comprised in the RNA strand. Further it is shown that if intercalator pseudonucleotides are positioned in relation to

179

each other, so that they are in close vicinity of each other when the oligonucleotides or oligonucleotide analogues are hybridized the melting temperature is decreased compared to when only the RNA strand comprises intercalator pseudonucleotides. 5 = amidite 5 from example 1 incorporated into the strand according to the procedure described herein above.

Example 13

10 **Fluorescence.**

A decreased fluorescence of the mono pyrene modified DNA strands upon binding to the complementary strands, indicates that the pyrene intercalates into the double helix. Double pyrene inserted oligonucleotides gives the same result for all of the different ODNs. This effect is more pronounced when the modified DNA is hybridized with ssDNA than when hybridized with ssRNA (Figures 5 and 6), indicating less intercalation of pyrene into the DNA/RNA Duplexes. This supports the conclusion from the thermal melting experiments about lacking of pyrene intercalation into the bulged DNA/RNA duplexes as deduced from the nearly identical melting temperatures with glycerol and pyrene bulges in the DNA/RNA duplexes (Table 3).

Two pyrene moieties separated by only one nucleotide generates a third peak at 480 nm, due to excimer formation of the pyrene residues. However this band is almost extinguished, when this type of DNA with two insertions with pyrene hybridizes to a complementary DNA strand. This indicates intercalation around an intact base-pair preventing the two pyrene moieties to get into the physical distance of approximately 3.4 nm needed for excimer formation. When a double inserted DNA hybridizes to a complementary RNA the two pyrene moieties are still able to interact since a substantial excimer band is found.

Example 14

3-Exonuclease stability of oligonucleotides or oligonucleotide analogues comprising intercalating pseudonucleotides

Materials and Methods:

5

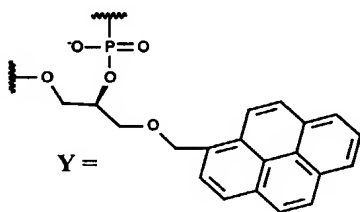
Time course of Snake Venom phosphodiesterase digestion of the DNA reference I, the INA oligo II and a mixture of both I and II. A 1.5 μ M solution of all the strands was use (1.5 μ M of each strand in the mixed assay) in 2 mL of buffer (0.1 M Tris-HCl; pH = 8.6; 0.1 M NaCl; 14 mM $MgCl_2$) was digested with 1.2 U Snake Venom phosphodiesterase (30 μ L of the following buffer solution: 5 mM Tris-HCl; pH = 7.5; 50% glycerol (v/v)] at room temperature.

10

DNA oligo: 3'-TGT CGA GGG CGT CGA

INA oligo: 5'- YAC AGC YTC CCY GCA GCY T

15



Results

20

The stability of an INA (Intercalating Pseudonucleotide comprised Nucleic Acid) oligonucleotide toward 3'-exonucleolytic degradation in vitro was evaluated and compared to normal DNA's stability using the Snake Venom phosphodiesterase (SVPDE), Figure 16. It is shown that the reference DNA oligonucleotide, I, is totally digested by SVPDE within 15 min. In contrast the INA oligonucleotide only shows a small hyperchromicity within the first 15 min. and thereafter no significant hyperchromicity is observed. These experiments indicates that the DNA nucleotide in the 3'-end of the INA oligonucleotide is digested by SVPDE, but when the enzyme meets the first intercalating pseudonucleotide it is stalled and unable to digest further.

30

The experiment where strand I and II are mixed in the SVPDE assay, giving a hybrid. A slow digestion compared to the reference DNA strand alone is observed. Almost a full degradation of the DNA strand is observed after 60 min. This result indicates that the hybrid, is degraded slower than the single stranded DNA.

5

Example 15

Hairpin shape oligonucleotides comprising intercalating pseudonucleotides for the detection of nucleic acid

10

Introduction

In this example it is shown how hairpin shaped oligonucleotides comprising intercalating pseudonucleotides (probe I) can be used for the detection of nucleic acids. It is further more shown that using this principle it is possible to detect as low as a 5 nM solution (1 pmol in 200 μ L) of target nucleic acid. It is also shown that the addition of Hexadecyl trimethyl ammoniumbromide (HTMAB) can enhance the signal sensitivity in a concentration dependent matter.

15

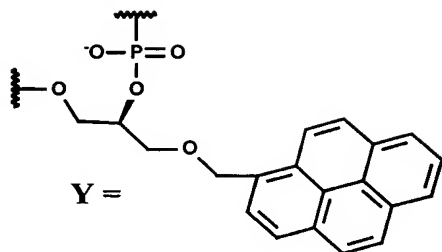
20

Materials and Methods

Below is shown the sequence of the detection probe comprising intercalating pseudonucleotides. The nucleotides which is involved in the hairpin formation is underlined and the nucleotides that are involved in the binding to target is in shown in **bold letters**:

25

Probe I: 5'- CAT CCG YAY **AAG CTT CAA** TCG GAT GGT TCT TCG



In figure FIGURE 17 is shown the secondary structure of the hairpin. The hydrogen bonds of the basepairs in the stem is shown as dots.

- 5 Below is shown the sequence of the target used in these experiments. The nucleotides participating in the binding of the detection probe is shown in **bold** letters:

Target: 3'- ATA GTA TTT ATT **CGA AGT TAG CCT ACC AAG AAG** CCT TTT TTG

- 10 All hybridisation experiments were carried out in a buffer solution containing:

140mM NaCl

10 mM Na₂HPO₄•2 H₂O

1 mM EDTA

- 15 pH = 7.0

The surfactant used in the experiments was HTMAB:

- 20 In Figure **FIGURE 18** is shown a figure that illustrates when the probe binds to its target sequence. It is shown that when the probe is hybridised to the Target, the two pyrene moieties from the intercalating pseudonucleotides are no longer separated by an intact base pair. This makes it possible for them to interact more freely, giving rise to higher excimer fluorescence:

25

In the table below is shown the designed of the experiment:

| Wells | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------|------------------|--------|----------------|---------------|-------------------|----------------|---------------|-------------------|
| a | H ₂ O | buffer | Probe I 100 | Probe I 10 | Probe I 1 pmol | Probe I 100 | Probe I 10 | Probe I 1 pmol |

183

| | | | pmol | pmol | | pmol + Tar- get 100 pmol | pmol + Tar- get 10 pmol | + Tar- get 1 pmol |
|----------|---|---|---|--|--|--|--|---|
| b | H ₂ O + sur- factant 10 ⁻⁶ | buffer + sufac- tant 10 ⁻⁶ | Probe I 100 pmol + sur- factant 10 ⁻⁶ | Probe I 10 pmol + sur- factant 10 ⁻⁶ | Probe I 1 pmol + sur- factant 10 ⁻⁶ | Probe I 100 pmol + Target 100 pmol + sur- factant 10 ⁻⁶ | Probe I 10 pmol + Target 10 pmol + sur- factant 10 ⁻⁶ | Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 ⁻⁶ |
| c | H ₂ O + sur- factant 10 ⁻⁵ | buffer + sufac- tant 10 ⁻⁵ | Probe I 100 pmol + sur- factant 10 ⁻⁵ | Probe I 10 pmol + sur- factant 10 ⁻⁵ | Probe I 1 pmol + sur- factant 10 ⁻⁵ | Probe I 100 pmol + Target 100 pmol + sur- factant 10 ⁻⁵ | Probe I 10 pmol + Target 10 pmol + sur- factant 10 ⁻⁵ | Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 ⁻⁵ |
| d | H ₂ O + sur- factant 10 ⁻⁴ | buffer + sufac- tant 10 ⁻⁴ | Probe I 100 pmol + sur- factant 10 ⁻⁴ | Probe I 10 pmol + sur- factant 10 ⁻⁴ | Probe I 1 pmol + sur- factant 10 ⁻⁴ | Probe I 100 pmol + Target 100 pmol + sur- factant 10 ⁻⁴ | Probe I 10 pmol + Target 10 pmol + sur- factant 10 ⁻⁴ | Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 ⁻⁴ |

All the probes and targets were annealed in 200 μ L buffer separately in Eppendorf tubes at 95°C for 2.5 min. and then slowly cooled to room temperature and trans-

184

ferred to a 96-well black plate from NUNC. The fluorescence was measured on a Wallac Victor², 1420 Multilabel counter, with the following specifications:

Emission filter: F340

5 Excitation filter: 500-10F

Measurement time: 0.1s, 4.0 mm from the bottom of the plate.

CW-lamp energy: 50054, Constant Voltage control.

Results and Discussion

10 Below is shown the results of the measurement:

| Wells | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------|------|------|--------|-------|------|--------|-------|------|
| a | 4299 | 3471 | 4927 | 3204 | 2639 | 20643 | 4988 | 3841 |
| b | 5709 | 3619 | 9684 | 4563 | 3644 | 18611 | 3971 | 4308 |
| c | 4021 | 3119 | 8429 | 1879 | 3456 | 13833 | 5337 | 2202 |
| d | 2236 | 3194 | 120959 | 12749 | 1898 | 223956 | 21684 | 4783 |

15 As can be seen from comparing **a3** with **a6**, there is a large increase in fluorescence on hybridisation of probe **I** to a target strand showing proofing the principle in using hairpin-shaped oligonucleotides for detection of nucleic acids sequences. If the background level (**a2**) is deducted from the measurement, nearly a 12 times increase in fluorescence of probe **I** upon hybridisation to its target sequence is observed.

20 By comparing **a4** with **a7** and **a5** with **a8** it can be seen that it is possible to detect the presence of as low as 10 down to 1 pmol of target nucleic acid.

25 The addition of surfactants on the fluorescence level is also shown. The addition of the HTMAB surfactant increases the fluorescence in some cases more than 100 times (column **6**), and hence increases the sensitivity of the detection up to a 100 times.

These results compared with the fact that probe **I** can be used as a primer in template directed extension reactions makes oligonucleotides or oligonucleotide ana-

logues a very useful tool in e.g. the detection of nucleic acids, for labelling nucleic acids, for the use in extension reactions like ligation and PCR and in real-time quantitative PCR.

5 Example 16

Control of oligobinding & INA-signal on SAL-chips.

Method

10

Chip production

- Oligos, 50 μ M, are spotted dissolved in 400 mM Sodium carbonate buffer, pH 9.
- The chip is instantly placed in humidity-chamber, 37° C for one hour.
- 15 • Oligo binding is effectuated on the surface of the chip by washing with 1% NH_4OH , 5 min.
- Unheated deionised water is used to wash for 2 x 2 min.
- The chips are centrifuged, 600 rpm for 5 min, to remove excess water from the surface.
- 20 • The chip is scanned, or stored refrigerated at 4° C.

SYBR Green II control staining of oligo-binding.

- Deionised water, approximately 90° C is used to wash for 2 x 2 min. Centrifugation, 600 rpm for 5 min follows.
- 25 • 10 000 x dilution of SYBR Green II is added to the chip, apply cover and incubate at ambient temperature for 2-3 min.
- Wash for 1 min, using unheated water and centrifuge the chip to dryness, 600 rpm for 5 min.
- The chip is now ready to be scanned, use Alexa 488 filterset.

30

Results

Section of chip from OUH, HUMAC, with amino-linker oligos stained with SYBR Green II.

See Figure **FIGURE 19**

5 Spot size: 100 μm . Center-center distance: 175 μm

Evaluation of oligo- and INA-binding on Asper SAL-chips (see Figure **FIGURE 20).**

10

- INA oligos with signal-modification and aminolinker, presumably bind to SAL chips like normal aminolinker oligos. (see SYBR Green Yes: 1,2,3,4 and 7).
- All signal-INAs fluoresce, when blue or blue-green filtersets are utilized on our ArrayWorx scanner (SYBR Green, No: 1, 2, 3, 4).

15

- The position of the signal-modification compared to the oligo-5'-end doesn't seem to be significant. No evident difference is observed between the strength of the signal depending whether the modification is furthest away from the 5'-end (1) or closest to the 5'-end. (4)

20

- The short 10-mer INAs, without linkers, apparently don't bind to the chip – they lack the linker (see 5). The signal is of the same strength as when clean buffer without oligos is spotted. (See SYBR Green Yes, 5 & 6)

- The variable background that is shown with different sections of the same chip can be caused by inadequate wash, calibration of scanner, or variation in SAL coating.

25

- Observed tendency: Generally the quality of spots, that is shape and signal-homogeneity, seem to be better, when the oligos contain INA modifications (compare 1 and 7, bottom right.)

Example 17

30

Hybridisation properties of DNA-DNA, INA-DNA and INA-INA hybrids at different pH values

Introduction

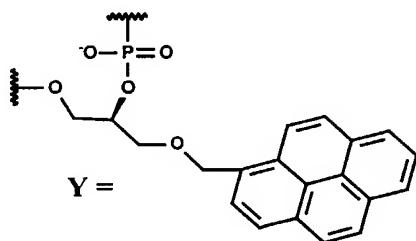
In this example it is shown the hybridisation affinities for DNA-DNA, INA-DNA and INA-INA hybrids at different pH values.

5

Materials and methods

Hybrids:

| | | |
|----|------------|--|
| 10 | Hybrid I | 5'-CTC AAC CAA GCT 3'-GAG TTG GTT CGA |
| | Hybrid II | 5'-CTC AAC YCA AGC T 3'-GAG TTG GTT CGA |
| 15 | Hybrid III | 5'-CTC AAC CAA GCT 3'-GAG TTG YGT TCG A |
| 20 | Hybrid IV | 5'-CTC AAC YCA AGC T 3'-GAG TTG YGT TCG A |



All hybridisation experiments were carried out with 1.5 μ M of both target and probe strands in 1 mL of a buffer solution containing:

25

140mM NaCl

10 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$

1 mM EDTA

30

The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of $\pm 1.0^\circ\text{C}$ as determined by repetitive experiments.

pH was adjusted with a solution of 25% NH_4OH and glacial acetic acid.

Results and discussion

| Hybrid # | pH 4.2 | 5.0 | 6.1 | 7.0 | 8.0 | 9.0 | 10.0 |
|----------|--------|------|------|------|------|------|------|
| I | 30.6 | 43.6 | 47.8 | 47.2 | 49.6 | 49.6 | 43.3 |
| II | - | 49.7 | 54.7 | 54.5 | 55.7 | 54.1 | 51.1 |
| III | - | 45.4 | 51.9 | 52.5 | 54.7 | 52.1 | 46.4 |
| IV | - | 36.1 | 46.0 | 46.5 | 48.5 | 46.5 | 40.9 |

In the table above is shown the results of the melting temperature experiments of hybrid I-IV at different pH values. As can be seen from the table hybrid II and III have higher melting temperatures over the pH range from pH = 5 to 10 than the homologous DNA duplex (hybrid I) and hybrid IV. This shows that it is possible to reduce the cross hybridisation between complementary sequences, when both sequences comprise at least one intercalating pseudonucleotide that are positioned opposite each other when said sequences hybridise. It can also be seen that the melting temperatures of all the hybrids are highest at around pH = 8, and hence in some preferred embodiments it is preferred to hybridise at pH = 8 ± 2 . The largest difference in melting temperature between the hybrids II and III comprising one intercalating pseudonucleotide and the hybrid IV comprising two opposite positioned intercalating pseudonucleotides is at pH = 5.0, namely 13.6°C and 9.3°C respectively. Hence in a preferred embodiment hybridisations between an oligonucleotide or oligonucleotide analogue comprising at least one intercalating pseudonucleotide and a nucleic acid or nucleic acid analogue is carried out at pH = 5 ± 1 .

Example 18

Preparing a sample for RT-PCR

5

The method of preparing a sample for RT-PCR of a target sequence is depicted in figure 7. The method has the advantage that false positive signals from DNA are largely reduced.

10 A cell sample is provided and the cell walls of the cell are destroyed, thereby releasing DNA and RNA from the cells (figure 7A). Subsequently, an oligonucleotide comprising an intercalator pseudonucleotide, which can hybridise to the target sequence is incubated with the DNA/RNA sample under conditions allowing hybridisation between the oligo and DNA (figure 7B). The sample is then ready to be up-
15 scaled by any standard RT-PCR procedure (figure 7C). Because target DNA present in the sample is blocked by hybridisation to the oligonucleotide, then only RNA may be amplified.

20 Alternatively, after destroying the cell walls, RNA may be purified by any standard method for example by extraction and precipitation (figure 7D). Usually, the purified RNA will comprise small amounts of DNA contamination. Hence, an oligonucleotide comprising an intercalator pseudonucleotide, which can hybridise to the target sequence is incubated with the RNA sample under conditions allowing hybridisation between the oligo and DNA (figure 7E). The sample is then ready to be up-
25 scaled by any standard RT-PCR procedure (figure 7F). Because target DNA contamination present in the sample is blocked by hybridisation to the oligonucleotide, then only RNA may be amplified.

Example 19

30

Preparing a sample for RT-PCR

190

The method of preparing a sample for RT-PCR of a target sequence is depicted in figure 8. The method has the advantage that false positive signals from DNA are largely reduced.

- 5 A cell sample is provided and the cell walls are destroyed, thereby DNA and RNA is released. RNA may be purified by any standard method from the sample (figure 8B), however it is also possible to perform the subsequent steps on the DNA/RNA sample.
- 10 The sample is incubated with beads linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 8C), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. After hybridisation the sample is filtered to remove the beads together with bound target DNA from the sample (figure 8D).
- 15 The sample is ready for RT-PCR (figure 8E). Because the sequence specific target DNA has been removed from the sample, the risk of false positives of the RT-PCR due to DNA contamination is largely reduced.
- 20 Alternatively, after sample preparation, the sample is incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 9B), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. After hybridisation the solid support is removed from the sample together with bound target DNA. The sample may once
- 25 again be incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide to remove traces of sequence specific DNA still left in the sample. The solid support is removed from the sample after hybridisation to sequence specific DNA (figure 9C).
- 30 The sample is then ready for RT-PCR.

Example 20

Purification of sequence specific DNA

The purification of sequence specific DNA is illustrated in figure 9 and 10.

5 A cell sample is treated with GnSCN thereby releasing nucleic acids. The sample is incubated with beads linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 10A), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. The sample is filtrated and washed to remove non-bound nucleic acids (figure 10B). The beads are subjected to heating and filtration, releasing pure, sequence specific DNA largely free of sequence specific RNA (figure 10C).

15 Alternatively, the nucleic acid sample is incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 11B), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. The solid support is separated from the rest of the sample and subjected to heating, which releases the sequence specific DNA (figure 11C). The sequence specific DNA will be largely free of sequence specific RNA and is ready for diagnosis, PCR or other purposes.

20 Example 21

Detection of target DNA

25 Oligonucleotides comprising pyrene pseudonucleotides are linked to a chip. The oligonucleotides are designed so that a part of it may hybridise to a specific target DNA and so that the oligonucleotide may also self-hybridise. When the oligonucleotide is hybridised to itself, 3 pairs of pyrene pseudonucleotides are facing each other, and accordingly the melting temperature of a DNA/oligo hybrid is higher than the melting temperature of the selfhybrid. Furthermore, the oligonucleotide comprises two pyrenes capable of forming an excimer, only when the probe is not hybridised to itself (figure 12 and figure 13A).

192

Different oligonucleotides recognising different target DNAs may be added to various defined regions of the chip. In the present example 2 different oligonucleotides are linked to spot 1 and spot 2, respectively.

- 5 A crude mixture of DNA fragments containing the target DNA is added to the chip at a temperature where the oligonucleotide can not selfanneal.

After an annealing step and a washing step, the temperature is lowered to allow self hybridisation of probes. Excimer formation is used to detect the presence of target
10 DNA as well as the amount of target DNA hybridised. The procedure is outlined in figure 12.

Alternatively, the oligonucleotide may be designed so that it comprises a fluorophore and a quencher, wherein the fluorophore signal may only be quenched by the
15 quencher when the oligonucleotide is self-hybridised (figure 13B).

It is also possible to use two oligonucleotides which each comprises 3 pyrenes pseudonucleotides that are facing each other when the oligonucleotides are hybridised. The oligonucleotides also contains a fluorophore and a quencher each, positioned so that the fluorophore signals may only be quenched by the quencher when
20 the oligonucleotides are hybridised (figure 13C).

Example 22

25 **Exciplex fluorescence from intercalating pseudonucleotides**

Introduction

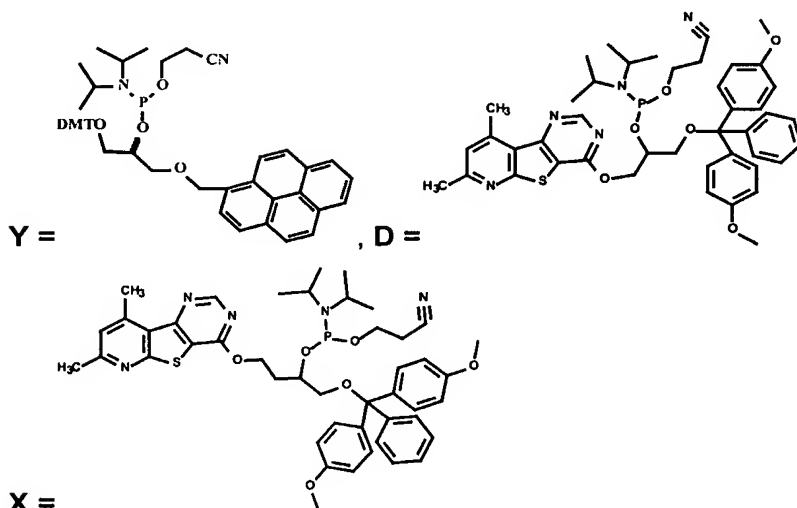
In this example is shown some exciplex fluorescence emission between two intercalating pseudonucleotides.
30

Materials, Methods and Results

193

Three oligonucleotides comprising two different intercalating pseudonucleotides were synthesized using standard procedures:

- Sequence 1: 5'-CTCAAYGDCAAGCT
 5 Sequence 2: 5'-CTCAAGYDCAAGCT
 Sequence 3: 5'-CTCAAGYXCAAGCT



- 10 After purification by HPLC the oligonucleotides comprising intercalating pseudonucleotides was dissolved in a buffer solution containing:

140mM NaCl

10 mM Na₂HPO₄•2 H₂O

- 15 1 mM EDTA

pH = 7.0

- and all fluorescence experiments were carried out in this buffer. Excitation was done at 343 nm on a Perkin Elmer MPF-3 spectrophotometer with at xenon 150 power supply.

- 20 As seen from Figures FIGURE 21 and FIGURE 22, the exciplex to monomer fluorescence ratio was higher when the to intercalating pseudonucleotides were positioned as neighbours (Figure FIGURE 21) than when placed as next-nearest neighbours (Figure FIGURE 22) - the exciplex transition was however clearly observed in both cases. Similar result was obtained with the amidite X.
- 25

As a conclusion an exciplex between two intercalating pseudonucleotides can be observed for both neighbouring and next-nearest neighbouring intercalating pseudonucleotides, when said pseudonucleotides are positioned internally in an oligonucleotide (See Figure FIGURE 23).

Example 23

PCR with oligonucleotide primers comprising intercalator pseudionucleotides

35 cycles of gradient PCR (94°C, 30 sec; gradient annealing temp, 45 sec; 72°C, 60 sec.) were performed with diluted plasmid template in a standard PCR-buffer (1.5 mM MgCl₂; 50 mM KCl; 10 mM Tris-HCl; 0.1% Triton X-100, 200 μ M of each dNTP, 5 pmol of each primer) in a final of volume of 25 μ l. PCR products were separated in a 0.7% agarose gel in 1xTBE buffer and visualized by EtBr staining. Temperatures on the figure 24 denote the annealing temperature in each well.

Primer designs (upstream and downstream, respectively)

| | | | |
|----|-----|-----|-----------------------------------|
| 20 | a01 | 5'- | AAGCTTCAATCGGATGGTTCTTCG |
| | a02 | 5'- | YAAGCTTCAATCGGATGGTTCTTCG |
| | a03 | 5'- | YCYATCCGAAAGCTTCAATCGGATGGTTCTTCG |
| | a05 | 5'- | CYAYTCCGAAAGCTTCAATCGGATGGTTCTTCG |
| 25 | b01 | 5'- | CACAAGAGCTGACCCAATGGTTGC |
| | b02 | 5'- | YCACAAGAGCTGACCCAATGGTTGC |
| | b03 | 5'- | YTYGGGTCACACAAGAGCTGACCCAATGGTTGC |
| | b05 | 5'- | TYGYGGTCACACAAGAGCTGACCCAATGGTTGC |

30 Primers 03 and 05 are able to form hairpin loops when not hybridized with target as exemplified below by the a05 primer:

| | | |
|----|--------------|-------------------------|
| 35 | Primer alone | TCAA |
| | | T TCGGA T GGTTCCTTCG-3' |

195
C AGCCTYAY
GAA C-5'

Primer 5'- CYAYTCCGA

5 AAGCTTCAATCGGATGGTTCTTCG
TTCGAAGTTAGCCTACCAAGAAGC
Target: 5'- TA

CT

10

Conclusion

As can be observed from the picture of the gel, the addition of one single end-positioned intercalator pseudonucleotide in the linear primers a02/b02 compared to the DNA control primers raises the effective melting temperature significantly.

From the amplification products of bands for the 03 and 05 primers it primers with beacon design primes PCR in a highly efficient manner.

20

Example 24

INA-oligo binding to DNA target

Results and discussion

Binding requires target-specificity and occurs spontaneously.

A series of INA-oligonucleotides were designed and tested for their abilities to spontaneously bind an 80 bp complementary target DNA sequence (Fig. 25).

Reactions were carried out by incubation of the double-stranded target DNA with an excess of P32-labelled INA-oligos (IOs) in a sodium-phosphate buffer containing 120 mM Sodium chloride at 37 °C for 1-3 hours. Results were then evaluated by electrophoretic mobility shift analysis and phosphor-imaging of the labelled IOs.

Figure 26 shows that all three IOs tested (IO 1-1, IO 1-2, IO 1-3) bound the target DNA. The relative amounts of bound IOs were determined by volume analysis of the retarded bands using the ImageQuant software. As the numbers at the bottom of the figure indicates the IOs showed different affinities for the target. The IO 1-3 clearly

had an advantage in binding the target and was therefore chosen for further analysis.

Evidence for the specificity of the observed binding was next ascertained. First P32 labelled IO 1-3 was incubated with either strand of the DNA target alone. As expected based on sequence complementarity IO 1-3 specifically bound the sense strand of the DNA target (Fig. 27, lane 1-3). Binding to the double stranded DNA target was then assayed at increasing concentration of P32 labelled IO 1-3 and compared to binding to an unrelated 60 bp target DNA sequence (compare lanes 4-5 with 7-8). Clearly, target binding by the IO required target complementarity as no binding was observed with the sequence-unrelated target DNA. To verify the position of the observed retardation a fraction of the target DNAs were P32 labelled and their retardation assayed in parallel (lanes 6 and 9).

IO pairing does not inhibit spontaneous binding and gives variable target-affinities by differential positioning of the intercalating units.

The observed target strand specificity of the IOs (ie. IO 1-3 specifically binding the sense strand of the target DNA, see above) suggested that the antisense strand of the target DNA may be free to be simultaneously attacked by a different IO. To explore this possibility IO 1-3 was annealed to three different, complementary IOs. As shown in figure 28 the pairing of the IOs still rendered the P32 labelled IO 1-3 capable of spontaneously binding the target DNA, albeit with different affinity depending on the positioning of the intercalating units in the pairing IO. As the IO 1-3 / IO 5 pair gave the best target-binding this pair was chosen for further testing. To investigate whether pairing affects the efficiency of spontaneous target binding, binding of IO 1-3 was assayed with and without previous pairing to IO 5. Figure 29 shows that pairing did not affect the spontaneous binding of IO 1-3 to the target DNA.

Nuclear factors aid IO target binding and favours paired IOs

RecA/Rad51 assisted joint molecule formation between DNA targets and small RNA-DNA oligonucleotides have previously been reported (Gamper 2000, Yoon 2002). The IO readily bound the target unassisted and thus hold promise as *bona fide* agents for DNA targeting for therapeutic purposes. It therefore was of great importance to clarify how this binding would proceed in a nuclear environment. To address this subject we employed nuclear extracts prepared from human cell culture.

As shown in figure 30, when reactions were carried out in the presence of nuclear extract retardation of IO 1-3 was at least 3-5 fold increased (calculations not shown), and only occurred in the presence of the specific target. Moreover, the degree of binding was dependent on the amount of nuclear protein added, and as such increased until a certain amount of protein was added. It then decreased as would be expected based on similar analysis with addition of protein involved in DNA repair processes. It is generally assumed that this process involves a D-loop formation of the targeted DNA, leaving both strands open for attack by matching oligonucleotides. It was therefore interesting to observe that upon addition of nuclear extract pairing IO's did indeed enhance the binding of the P32-labelled IO 1-3 to the DNA target (Figure 31).

Materials and Methods

Oligo-synthesis: all oligos were prepared by standard procedures.

Radioactive labelling of oligos: oligos were endlabelled by incubation with polynucleotide kinase and γ -P³² ATP. Labelled oligoes were purified by the Mermaid kit procedure.

Nuclear extracts: HT-29 extracts were prepared from pre-confluent HT-29 colon-cancer cells by the NUN extraction procedure. HeLa nuclear extracts were obtained from (a company in Belgium).

Electrophoretic mobility assay: Reactions were carried out in 20 mM sodium-phosphate buffer pH 8.0 containing 120 mM NaCl, 1 mM DTE. Upon incubation at 37°C reactions were snap-frozen in liquid N₂ and stored at -80°C or applied directly to electrophoresis on 7 or 10 % polyacrylamide gel cast in ½ x TBE, at 300 V for 2-4 h, at 4°C.

Reactions containing nuclear extracts additionally contained 0.22M Urea, a total of 200mM NaCl, 0.22 % NP-40, 5.52 HEPES, 5 mM MgCl₂ and 2 mM ATP. These reactions were incubated at 37°C for 10 min. upon which 1.175 µl 10% SDS and 37.5 µg Proteinase K was added and incubation reassumed for another 60 min.

For comparative purposes equal CPM of individual IOs were added to reactions.

Visualisation: EMSA results were evaluated using a STORM phosphorimager and the ImageQuant software.

Example 25

5

LNA + INA: Making locked nucleic acid hairpins accessible to targeting by insertion of intercalating nucleic acid monomers

10 INTRODUCTION

Hairpin structures are a common feature of single-stranded DNA and RNA sequences. This type of secondary structures can make target sequences inaccessible to intermolecular Watson-Crick base pairing (ⁱ). There is a need to find new techniques to alleviate this problem. The ones previously reported are using fragmentation of the nucleic acid sequence close to the target (ⁱⁱ) or replacing natural 2'-deoxycytidine with *N*⁴-ethyl-2'-deoxycytidine which forms base pairs with 2'-deoxyguanosine having reduced stability as compared with natural base pairs (ⁱⁱⁱ). A variety of modified oligonucleotides have been developed during the last two decades in order to develop potential gene inhibitors which possess an enhanced stability towards cellular nucleases, the ability to penetrate the cell membrane and an efficient hybridisation to the target RNA/DNA. If the modified oligonucleotides have efficient hybridisation properties, they are also expected to form secondary structures which can make a large number of sequences inaccessible, but to our knowledge no attempts have been done on modified oligonucleotides to overcome this problem.

LNA oligonucleotides are oligonucleotides containing a conformationally restricted monomer with a 2'-O, 4'-C-methylene bridge (Fig. 1) and they have shown helical thermal stability when hybridised to either complementary DNA or RNA when compared with unmodified duplexes. Due to their hybridisation efficiencies they are also expected to form extremely stable hairpin structures. It is challenging also to make LNA hairpin structures accessible to targeting as LNA seems to be the most promising antisense candidate among modified oligonucleotides.

INAs (Intercalating Nucleic Acids, Fig. 1) composed by insertions of intercalating pseudonucleotides into DNA are strongly discriminating between DNA

over RNA when hybridising with them (^{iv}). Properly designed INAs gives more stable INA/DNA duplexes than its DNA/DNA counterparts whereas the opposite is found when INAs are hybridising to RNA which results in less stable duplexes than the corresponding RNA/RNA duplexes. In this paper it is shown that this property can be used to make LNA hairpins more accessible to targeting of DNA by inserting INA monomers into the stem of the hairpin.

MATERIALS AND METHODS

Synthesis of DMT protected LNA and INA phosphoramidites

The phosphoramidite of LNA and INA, respectively, were prepared as previously described (4a,8a).

ODN, LNA and INA synthesis, purification and measurement of melting temperatures.

The ODN, LNA and INA synthesis was carried out on an Expedite™ 8909 Nucleic Acid Synthesis System from Applied Biosystems. The LNA and INA amidite was dissolved in a 1:1 mixture of dry acetonitrile and dry dichloromethane, as a 0.1 M solution, and inserted into the growing oligonucleotides chain using same conditions as for normal nucleotide couplings (2 min coupling). The coupling efficiency of the modified nucleotide was > 99%. The ODNs, LNAs and INAs were synthesised with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-1 column. Buffer A: 950 ml of 0.1 M NH₄HCO₃ and 50 ml MeCN, pH 9.0; buffer B: 250 ml of 0.1 M NH₄HCO₃ and 750 ml MeCN, pH 9.0. Gradients: 5 min 100% A, linear gradient to 100% B in 40 min, 5 min with 100% B, linear gradient to 100% A in 1 min and then 100% A in 29 min (product peak at ~ 37 min). The ODNs, LNAs and INAs were DMT deprotected in 925 µl of H₂O and 75 µl CH₃COOH and purified by HPLC, again using the same column, buffer system and gradients (product peak at ~ 26 min). To get rid of the salts, the ODNs, LNAs and INAs were redissolved in 1 ml of water and concentrated *in vacuo* three times.

All ODNs, LNAs and INAs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and PECSS software package v. 4.3. All ODNs were measured in a 120 mM NaCl,

200

10 mM, sodium phosphate, 1 mM EDTA, pH 7.0, 3.0 μ M each strand. All melting temperatures are with an uncertainty $\pm 0.5^\circ\text{C}$ as determined by repetitive experiments.

5 RESULTS AND DISCUSSION

Duplexes

NMR has been used to determine the structure of DNA/LNA duplexes and it was found that only one LNA monomer in the duplex was sufficient to induce a change in the sugar conformation of the flanking nucleotids from a north conformation typically found in B-type DNA/DNA duplexes to a south conformation, the latter being the one typically found in the sugar parts of A-type RNA/RNA duplexes (γ). It was therefore found interesting to investigate systematic insertions of the INA monomer **P** (Fig. 32) at all possible sites of this duplex (Figure 33). The oligo **I** without any LNA monomers was used as a reference target for the hybridisations with the probes 2-12 (Figure 33) having insertions of **P** at all possible positions, except at the 3'-end. The oligo **I** had in all cases increased duplex stabilities when compared with the unmodified probe 1. In fact, probe 3 gave a remarkably stable duplex with an increase of 10.1 $^\circ\text{C}$ in the thermal melting temperature. This oligo has the INA monomer **P** inserted in an AT region and when compared with the other probes, this seems generally preferable.

For the oligo **II** it was observed that insertions of the INA monomer **P** into regions of its duplexes away from the LNA monomer increased the duplex stability when compared with the unmodified probe 1. In fact the stabilisations were nearly identical with those observed for the oligo **I** and this confirms that regions away from the LNA monomer have still a B-type structure. Only when the insertion of **P** was done into the complementary oligo close to the LNA monomer (Figure 33, entries 2-5), differences could be observed in hybridisation efficiencies between the two oligos upon hybridisation to their complementary sequences. This could be ascribed to a conformational change of the sugar part of the neighbouring nucleotides and this was reflected by a decrease in thermal melting temperatures. The major differences were found for the duplexes with neighbouring insertions to the LNA monomer (Figure 33, entries 2 and 3) whereas minor differences were found for duplexes with next neighbouring insertions (Figure 33, entries 4 and 5).

Oligo **III** with three evenly placed LNA monomers is supposed to induce A type duplex structure in most parts of the duplex formed on its hybridisation

with its complementary sequence. This is deduced by comparing ΔT_m s for the oligo III with those for the oligo I. The conclusion is in agreement with NMR structure determination on a similar duplex with three LNA monomers (^v). Comparable stabilisations were only found for these two oligos when the INA monomer **P** was inserted close to the ends of their respective duplexes and thus confirming B-type duplex at the ends (Figure 33, entries 10 and 11).

The study on the oligos I-III demonstrates INA monomer **P** insertions as a versatile tool of distinguishing A and B type duplex regions when a modified nucleotide induces an A type duplex structure into a region of a B type duplex.

Hairpins

On heating the hairpin forming oligo T₄-DNA (see Figure 34 for sequence key) alone in a thermal melting experiment a clear transition is observed at 37.2 °C (Figure 2A and Figure 34) which is ascribed to opening of the hairpin to an ssDNA. In a similar experiment with T₄-LNA (Figure 34) a higher transition temperature is to be expected for opening of the corresponding hairpin due to higher stability of the stem which is deduced from the reported higher stabilities of LNA/DNA duplexes. To test this hypothesis T₄-LNA analogous to T₄-DNA was synthesised with five nucleotides in the stem being replaced with the corresponding LNA monomers (T^L and MeC^L, respectively). For this modified hairpin only an incipient transition from hairpin to ssLNA could be observed above 80 °C (Fig. 35A). An equimolar mixture of T₄-DNA and A₄-DNA gives a transition at the same temperature as the one observed for T₄-DNA alone, but the increase of optical density (hyperchromacia) is much stronger for the transition of the A₄-DNA/T₄-DNA mixture. The increase in hyperchromacia of the mixture is not due to an additional transition of an A₄-DNA hairpin because this oligo alone has no transition above 20 °C which is in agreement with earlier reports that adenine compared to thymine in the loop destabilises a hairpin (^{vi}). The increased hyperchromacia of the mixture is therefore best explained by the melting of an A₄-DNA/T₄-DNA duplex although it is impossible to estimate the ratio of distribution of T₄-DNA between its hairpin structure and its duplex structure with A₄-DNA.

Due to the stability of the T₄-LNA hairpin and the instability of the A₄-DNA hairpin no transitions were expected to be found in the temperature range 20–80 °C for a mixture of these two oligos. It was therefore puzzling to find a transition at 37.3 °C with a rather low hyperchromacia. The extra transition for the A₄-DNA/T₄-LNA mixture is best understood by comparison with the properties of palin-

dromic sequences which have been extensively studied by NMR. For example, it was shown that the self complementary sequence 5'-CGCGTTAACGCG formed a duplex at lower temperatures with a transition to a hairpin at 33 °C at 0.3 mM and again a transition to random coil at 48 °C ^(vii). As depicted in Figure 36, Scheme 1

5 our system is nearly the same, except that the hairpin forming oligo (T₄-LNA) does not have a self complimentary sequence in the loop region and needs another oligo to form the duplex. In our case the required oligo (A₄-DNA) does not form a hairpin at ambient temperatures and is therefore not introducing any complications for the interpretation of the melting of the A₄-DNA/T₄-LNA mixture. By comparison with the

10 nature of the palindromic sequences the melting at 37.3 °C of the A₄-DNA and T₄-LNA mixture is best explained by a transition from DNA/LNA duplex to a mixture of A₄-DNA and T₄-LNA hairpin. For palindromic oligos it has been suggested that the transition from duplex to hairpin takes place through formation of a cruciform structure formed after creation of an initial bulge in the center of the duplex upon melting

15 ^(viii). Once the cruciform is formed little energy is needed to propagate the mobile junction formed and to complete the separation of the two hairpins. We can argue for a similar mechanism in our case and also for the same type of mechanism operating in the opposite direction, because identical melting curves for up and down temperature modes were obtained. This could implicate that we have found an ex-

20 ample of strand invasion into an extremely stable LNA hairpin.

For the 5'-CGCGTTAACGCG sequence, hairpin structures were always observed by NMR at lower temperatures and complete conversion from hairpin to duplex was never observed ^(vii) which may indicate quenching of the equilibrium at temperatures lower than the transition temperature. Also in our case a rather

25 low hyperchromacia for the transition seems to indicate that the conversion from a mixture of A₄-DNA and T₄-LNA hairpin to DNA/LNA duplex is incomplete. This implies a more complete transition from hairpin to duplex if the melting temperature is higher and closer to the melting of the LNA hairpin. This is indeed what we found when a pyrene pseudonucleotide is inserted in the middle of the A₄ region in the A₄-

30 DNA (Figure 34). It has previously been found that a single INA insertion in an A/T region of a duplex causes a significant increase in the melting temperature. This is also observed here with a melting of 44.7 °C, and furthermore, a significant increase in the hyperchromacia is observed when compared with the DNA/LNA duplex from A₄-DNA (Figure 2A and 2D). The higher hyperchromacia in this case indicates that

the A₂PA₂-DNA/T₄-LNA duplex has a better ability to be formed in the transition from the LNA hairpin.

From the finding above that pyrene insertions opposite to the LNA monomer lower the melting temperatures of LNA containing duplexes, we tested that proper insertions in the stem of a T₄-LNA hairpin could reduce its stability and make it prone to targeting to A₄-DNA. As the transition temperature of T₄-LNA is too high to be determined, it was promising to observe a thermal transition for the mono pyrene inserted oligos P²-LNA and P⁵-LNA at 81.1 °C and 71.4 °C, respectively, and for the double pyrene inserted oligo P-P-LNA at 69.1 °C though it has to be admitted that the hyperchromacia was extremely low for the three transitions. This means that one shall be very cautious about the interpretation and this is symbolised by using parentheses for these transitions in Figure 34. Irrespectively whether the transitions are due to opening of hairpins or to undefined duplex meltings, we took it as evidence for assuming that these pyrene inserted oligos could be more accessible for duplex formation with their complementary ssDNA targets.

When the oligo P²-LNA with the INA monomer **P** was inserted after the first nucleotide in the stem, a considerably stronger hyperchromacia was observed on melting of the duplex with A₄-DNA (Fig. 35B) than for T₄-LNA with A₄-DNA without any insertion of **P** (Fig. 35A). The hyperchromacia is approximately half of the one observed for the T₄-DNA/A₄-DNA duplex which is shown in Figs 2A-C as a reference. An increase in the transition temperature is also observed as should be expected because of the stabilising effect of **P** on hybridisation to a DNA. For both the transition temperature and hyperchromacia a similar result is found for the oligo P⁵-LNA with a **P** insertion close to the loop of its corresponding hairpin form (Fig. 35C). With two **P** insertions in the stem region the resulting oligo P-P-LNA shows an even higher transition temperature for its corresponding duplex with A₄-DNA, but more strikingly, the hyperchromacia is nearly the same as the one for the unmodified duplex. This is a clear demonstration that **P** insertions into LNA with secondary structures can make this special type of LNA more accessible to targeting and at the same time increase the duplex stability with the target, the latter being deduced from higher transition temperatures for the LNA probes with **P** insertions. It was attempted further to stabilise the duplexes with the LNA probes by inserting **P** into the target in the region corresponding to the loop in the probes. As seen from Figure 34, the oligo A₂PA₂ showed even higher transition temperatures and again the highest one was found for two **P** insertions in the LNA probe (P-P-LNA). Stabilising the du-

plexes by extra insertions in the target also improved the hyperchromacia as it is seen for the A₂PA₂ oligo (Fig. 35D). The highest melting temperature was found for (APA)₂-DNA when forming a duplex with P-P-LNA. In this case the melting temperature is considerably higher (83.3 °C) than the transition temperature (69.1 °C) measured for what is most likely the P-P-LNA hairpin.

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Example 26**Preparation of an intercalator pseudo nucleotide**

The example describes preparation and use of *N*-(pyrene-1-ylmethyl)-(3*R*,4*R*)-4-(hydroxymethyl)pyrrolidin-3-ol (**4**) in the synthesis of several INAs and investigated the hybridisation affinity of INA/DNA, INA/RNA duplexes and DNA TWJ region. When the *N*-(pyren-1-ylmethyl)azasugar was inserted as a bulge good discrimination between stabilities of INA/DNA and INA/RNA duplexes and the increased stability of a DNA three-way junction were observed.

The synthesis of 1'-aza pyrenemethyl pseudonucleoside **4** started from enantiomerically pure 1-aza analogues of 2-deoxy-D-hexofuranose **1** or 2-deoxy-D-ribofuranose **2**.¹³ Pyrene substrates having chloromethyl and carbaldehyde functionalities that could be coupled with the secondary amines **1** and **2** were used (Fig. 37).

The DMT protected phosphoramidite **6** is required for the oligonucleotide synthesis. The primary alcohol **4** was treated with an excess of DMTCl in pyridine with further purification on a silica gel column to give compound **5** in 61% yield. The synthesis of the final phosphoramidite by treatment with 2-cyanoethyl-*N,N*-isopropylchlorophosphoramidite in the presence of the excess of Hunig's base¹² failed. To obtain the required phosphoramidite **6** we used an alternative method with 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane and *N,N*-diisopropylammonium tetrazolide.¹⁵ The compound **6** was obtained in 57% yield.

The phosphoramidite **6** was incorporated into different oligonucleotide sequences to give INAs on an automated solid phase DNA synthesizer using an increased coupling time (24 min) and repeating the cycle twice. The coupling efficiencies for the pyrene azasugar derivative **6** were approximately 80-85% compared to approximately 99% for commercial phosphoramidites (2 min coupling).

The synthesised INAs were used in the hybridisation studies of INA/DNA and INA/RNA duplexes (Fig. 38) and INA/DNA three way junction (TWJ) (Fig. 39).

INA with incorporation of the *N*-(pyren-1-ylmethyl)azasugar as the bulge resulted in lowering of the melting temperature with 1.2 °C per modification towards ssDNA (Fig. 15). The corresponding reference duplex in entry **B** containing a bulging de-

oxynucleotide (dG) had a considerably lower $T_m = 32.2\text{ }^{\circ}\text{C}$ ($\Delta T_m = -10.8\text{ }^{\circ}\text{C}$). For the INA/RNA duplexes the pyrene containing sequence **C** and the reference **B** decreased the stability of the INA/RNA duplex with $10\text{ }^{\circ}\text{C}$ and $9.6\text{ }^{\circ}\text{C}$, respectively, compared to the perfectly matched duplex (entry **A**). Consequently, INA with pyrene azasugar incorporated as the bulge has better hybridisation affinity towards the complementary ssDNA than towards ssRNA. The differences in melting temperatures for ssDNA and ssRNA seems to be additive with respect to the number of pyrene moieties in the targeting ODN. These results are also in agreement with other investigations where 1-O-(1-pyrenylmethyl)glycerol was inserted twice as bulges. A larger discrimination up to $25.8\text{ }^{\circ}\text{C}$ between INA/DNA and INA/RNA was then observed.⁸ In that case the INA/DNA structure is stabilised compared to the wild type duplex in contrast to our case where a slight decrease is observed for T_m . The flexibility of the bulge may be an important factor to obtain both duplex stabilization and discrimination. The synthesis of different linkers and planar aromatic moieties is also in progress. The RNA/DNA discrimination displayed may be applied for purification or detection of DNA targets in a mixture with the very same sequences of RNA.

DNA three way junction (TWJ) composed of two arms linked to a stem (Fig. 39), was observed to lead to a considerable stabilisation when the pyrene azasugar intercalator was inserted in the INA (**F3**) compared to the ODN having dA at the same position (**F2**) or without an insertion in the ODN (entry **F1**). To be sure that hybridization in the arms is important for the stability of the complex; we prepared ODNs with mismatches in either arm of TWJ (entry **E2** and **E3**). In both cases it resulted in a large lowering of the hybridisation affinity.

Experimental

General

NMR spectra were recorded on a Bruker AC-300 FT NMR spectrometer at 300 MHz for ^1H NMR and at 75.5 MHz for ^{13}C NMR. Internal standards used in ^1H NMR spectra were TMS (δ : 0.00) for CDCl_3 , CD_3OD ; in ^{13}C NMR were CDCl_3 (δ : 77.0), CD_3OD (δ : 49.0). Accurate ion mass determination was performed on a Kraton MS-50-RF equipped with FAB source. The $[\text{M}+\text{H}]^+$ ions were peakmatched using ions derived from the glycerol matrix. Thin layer chromatography (TLC) analyses were carried out with use of TLC plates 60 F₂₅₄ purchased from Merck and were visual-

ized in an UV light (254 and/or 343 nm) and/or with a ninhydrin spray reagent (0.3 g ninhydrin in 100 cm³ butan-1-ol and 3 cm³ HOAc) for azasugars and its derivatives. The silica gel (0.063-0.200) used for column chromatography was purchased from Merck. ODNs were synthesised on an Assembler Gene Special DNA-Synthesizer (Pharmacia Biotech). Purification of 5'-O-DMT-on and 5'-O-DMT-off ODNs were accomplished using a Waters Delta Prep 4000 Preparative Chromatography System. The modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. All solvents were distilled before use. The reagents used were purchased from Aldrich, Sigma or Fluka. The reagents for Gene Assembler were purchased from Cruachem (UK).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*S*)-4-[(1*S*)-1,2-dihydroxyethyl]pyrrolidin-3-ol (3)**

Method A. Azasugar **1** (50 mg, 0.34 mmol) was dissolved in DMF (5 cm³), 1-(chloromethyl)pyrene (103 mg, 0.41 mmol) and Et₃N (0.057 cm³, 0.41 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm³). The residue was chromatographed on a silica gel column with CH₂Cl₂/MeOH (0-20%, v/v) as eluent affording the pure product **3** (70 mg, 57%): *R*_f 0.20 (10% MeOH/CH₂Cl₂); δ_H(CD₃OD) 2.36 (1 H, m, H-4), 2.95 (1 H, m, H-5), 3.08 (1 H, dd, *J* 2.8 and 10.5, H-2), 3.22 (1 H, dd, *J* 5.4 and 12.0, H-5), 3.34 (1 H, m, H-2), 3.50-3.65 (3 H, m, CH[OH]CH₂OH), 4.42 (1 H, m, H-3), 4.65 (2 H, s, CH₂pyren-1-yl), 4.88 (3 H, br. s, 3×OH), 7.90-8.40 (9 H, m, H_{arom}); δ_C(CD₃OD) 50.7 (C-4), 56.7 (C-5), 57.2 (C-2), 62.7 (CH₂pyren-1-yl), 65.7 (CH₂OH), 71.8 (CH[OH]), 72.7 (C-3), 123.8, 125.5, 125.8, 125.9, 126.6, 126.8, 127.3, 128.0, 128.2, 129.1, 129.4, 129.9, 131.2, 131.9, 132.5, 133.2 (pyren-1-yl); *m/z* (FAB) 362.1748 [M+H]⁺, C₂₃H₂₄NO₃ requires 362.1756.

Method B. Azasugar **1** (70 mg, 0.48 mmol) was dissolved in DMF/EtOH (3:1, 10 cm³) and 1-pyrenecarbaldehyde (270 mg, 1.18 mmol) and NaCNBH₃ (74 mg, 1.18 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. Solvent was evaporated under reduced pressure, co-evaporated with toluene (2×5 cm³). The residue was purified using silica gel column chromatography with CH₂Cl₂/MeOH (0-20%, v/v) affording the compound **3** (110 mg, 63%).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-(hydroxymethyl)pyrrolidin-3-ol (4)**

Method A. Azasugar **2** (100 mg, 0.86 mmol) was dissolved in DMF (10 cm³) and 1-(chloromethyl)pyrene (257 mg, 1.03 mmol) and Et₃N (0.140 cm³, 1.03 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm³). The residue was dissolved in H₂O/CH₂Cl₂ (1:1, 40 cm³) and the water layer was extracted with CH₂Cl₂. The combined organic fractions were dried (Na₂SO₄), evaporated *in vacuo* and chromatographed on a silica gel column with CH₂Cl₂/MeOH (0-20%, v/v) affording the title compound **4** (130 mg, 46%): *R*_f 0.17 (10% MeOH/CH₂Cl₂); δ_H(CDCl₃) 2.08 (1 H, m, H-4), 2.29 (1 H, m, H-5), 2.57 (1 H, dd, *J* 2.8 and 10.2, H-2), 2.85 (2 H, m, H-2 and H-5), 3.43 (2 H, s, CH₂OH), 3.47 (2 H, br. s, 2×OH), 4.08 (1 H, m, H-3), 4.19 (2 H, s, CH₂pyren-1-yl), 7.90-8.40 (9 H, m, H_{arom}); δ_C(CDCl₃) 49.8 (C-4), 55.9 (C-5), 57.5 (C-2), 62.3 (CH₂pyren-1-yl), 64.2 (CH₂OH), 73.9 (C-3), 123.3, 124.4, 124.6, 124.8, 125.1, 125.9, 127.3, 127.6, 127.8, 129.5, 130.7, 130.9, 131.1 (pyren-1-yl); *m/z* (FAB) 332.1631 [M+H]⁺, C₂₃H₂₄NO₃ requires 332.1651.

Method B. Azasugar **2** (1.18 g, 10.1 mmol) was dissolved in DMF/EtOH (3:1, 150 cm³) and 1-pyrene-carbaldehyde (3.47 g, 15.1 mmol) and NaCNBH₃ (950 mg, 15.1 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×50 cm³). The residue was dissolved in H₂O/CH₂Cl₂ (1:1, v/v, 150 cm³) and the water layer was extracted with CH₂Cl₂ (3×75 cm³). The combined organic fractions were dried (Na₂SO₄), evaporated under diminished pressure. The residue was purified using silica gel column chromatography with CH₂Cl₂/MeOH (0-20%, v/v) affording the compound **4** as an oil which crystallised on standing (1.9 g, 57%), mp 104 - 105 °C.

Method C. A cooled solution of compound **3** (110 mg, 0.304 mmol) in EtOH (4 cm³) was added to a solution of NaIO₄ (71.6 mg, 0.335 mmol) in H₂O (1.5 cm³) under stirring. After 30 min NaBH₄ (12.3 mg, 0.335 mmol) was added. After 30 min the resulting solution was acidified with 2M HCl until pH 2 under vigorous stirring. The solvent was removed *in vacuo*. The residue was dissolved in H₂O/CH₂Cl₂ (1:1, v/v, 20 cm³) and extracted with CH₂Cl₂ (4×15 cm³). The combined organic layers were

dried (Na_2SO_4), evaporated under diminished pressure to dryness affording compound **4** (40 mg, 40%).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidin-3-ol (**5**)**

Compound **4** (139 mg, 0.42 mmol) was dissolved in anhydrous pyridine (10 cm³) and DMTCl (178 mg, 0.53 mmol) was added. The mixture was stirred for 24 h under nitrogen at room temp. MeOH (1 cm³) was added to quench the reaction and the solvents were evaporated under reduced pressure and co-evaporated with toluene (2×5 cm³). The residue was re-dissolved in $\text{H}_2\text{O}/\text{CH}_2\text{Cl}_2$ (1:1, v/v, 20 cm³), and the mixture was washed with saturated aqueous NaHCO_3 . The organic layer was dried (Na_2SO_4), and concentrated under reduced pressure. Purification using silica gel column chromatography (5-40% EtOAc/cyclohexane, v/v) gave the title compound **5** as a foam (160 mg, 61%) which was used in the next step without further purification: R_f 0.45 (49% EtOAc/49% cyclohexane/2% Et_3N , v/v/v); δ_{H} (CDCl_3) 2.20 (1 H, m, H-4), 2.34 (1 H, m, H-5), 2.53 (1 H, br.s, OH), 2.62 (1 H, dd, J 5.6 and 9.9, H-2), 2.72 (1 H, dd, J 2.5 and 9.8, H-2), 3.06 (3 H, m, CH_2ODMT and H-5), 3.71 (6 H, s, OCH_3), 4.01 (1 H, m, H-3), 4.21 (2 H, s, $\text{CH}_2\text{pyren-1-yl}$), 6.78 (4 H, m, DMT), 7.10-7.40 (9 H, m, DMT), 7.90-8.40 (9 H, m, H_{arom}); δ_{C} (CDCl_3) 48.8 (C-4), 55.2 (OCH_3), 56.1 (C-5), 58.0 (C-2), 61.9 ($\text{CH}_2\text{pyren-1-yl}$), 64.5 (CH_2OH), 74.9 (C-3), 85.9 (C- Ar_3), 113.0, 123.8-132.3 (DMT and pyren-1-yl), 144.9, 158.4 (DMT); m/z (FAB) 634.2740 $[\text{M}+\text{H}]^+$, $\text{C}_{44}\text{H}_{42}\text{NO}_5$ requires 634.2722.

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidine (**6**)**

Compound **5** (140 mg, 0.22 mmol) was dissolved under nitrogen in anhydrous CH_2Cl_2 (5 cm³). *N,N*-Diisopropylammonium tetrazolide (61 mg, 0.42 mmol) was added followed by dropwise addition of 2-cyanoethyl-*N,N,N',N'*-etraisopropylphosphane (0.140 cm³, 0.44 mmol). After 2.0 h analytical TLC showed no more starting material and the reaction was quenched with H_2O (1 cm³) followed by addition of CH_2Cl_2 (10 cm³). The mixture was washed with saturated aqueous NaHCO_3 (2×10 cm³). The organic phase was dried (Na_2SO_4) and the solvents were removed under reduced pressure. The residue was purified using silica gel column chromatography with cyclohexane/EtOAc (0-20%, v/v). Combined UV-active fractions were evapo-

rated *in vacuo* affording **6** (158 mg, 57%) as foam that was co-evaporated with dry acetonitrile (3×30 cm³) before using it in ODN synthesis. *R*_f 0.85 (49% EtOAc/49% cyclohexane/2% Et₃N, v/v/v); δ_{H} (CDCl₃) 0.93 (6 H, m, CH₃ [Prⁱ]), 1.04 (6 H, m, CH₃ [Prⁱ]), 2.30 (2 H, m, H-4 and H-5), 2.48 (2 H, m, CH₂CN), 2.64 (1 H, m, H-2), 2.78 (1 H, m, H-2), 2.98 (2 H, m, OCH₂CH₂CN), 3.08 (1 H, m, H-5), 3.50 (4 H, m, CH [Prⁱ] and CH₂ODMT), 3.65 (6 H, s, OCH₃), 4.01 (1 H, m, H-3), 4.20 (2 H, m, CH₂pyren-1-yl), 6.68 (4 H, m, DMT), 7.05-7.40 (9 H, m, DMT), 7.85-8.40 (9 H, m, H_{arom}); δ_{P} (CDCl₃) 148.2 (s), 149.0 (s) in the ratio 2:1.

Synthesis and purification of modified and unmodified oligodeoxynucleotides

The oligodeoxynucleotides were synthesised on a Pharmacia Gene Assembler[®] Special synthesizer in 0.2 μmol -scale (7.5 μmol embedded per cycle, Pharmacia primer supportTM) using commercially available 2-cyanoethylphosphoramidites and **6**. The synthesis followed the regular protocol for the DNA synthesizer. The coupling time for **6** was increased from 2 to 24 min and the cycle was repeated twice. The 5'-O-DMT-on ODNs were removed from the solid support and deprotected with 32% aqueous NH₃ (1 cm³) at 55 °C for 24 h and then purified on preparative HPLC using a Hamilton PRP-1 column. The solvent systems were buffer A [950 cm³ 0.1 M NH₄HCO₃ and 50 cm³ CH₃CN (pH = 9.0)] and buffer B [250 cm³ 0.1 M NH₄HCO₃ and 750 cm³ CH₃CN (pH = 9.0)] which were used in the following order: 5 min A, 30 min liner gradient of 0-70% B in A, 5 min liner gradient of 70-100% B in A. Flow rate was 1 cm³ min⁻¹. The purified 5'-O-DMT-on ODNs eluted as one peak after approximately 30 min [UV control 254 nm and 343 nm (for pyrene containing ODNs)]. The fractions were concentrated *in vacuo* followed by treatment with 10% aqueous HOAc (1 cm³) for 20 min and further purification on HPLC under the same conditions to afford detritylated ODNs which eluted at 23-28 min. The purity of oligos synthesised was 99-100% according to the preparative HPLC. The resulted solutions were evaporated *in vacuo* and co-evaporated twice with water to remove volatile salts to afford ODNs, which were used in melting temperature measurements. All oligonucleotides containing pyrenylmethylazasugar derivative **6** were confirmed by MALDI-TOF analysis (entry **C**: found 4005.65, calcd. 4005.76; entry **D**: 4398.02, calcd. 4398.87; entry **F3**: found 4903.05, calcd. 4904.89).

Melting experiments

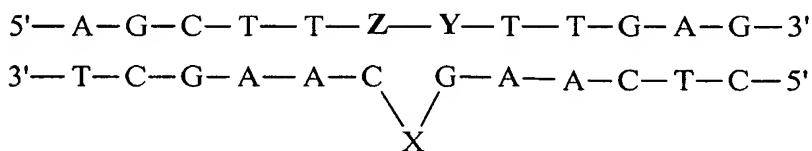
Melting temperature measurements were performed on a Perkin-Elmer UV/VIS spectrometer fitted with a PTP-6 Peltier temperature-programming element. The absorbance at 260 nm was measured from 18 °C to 85 °C in 1 cm cells. The melting temperature was determined as the maximum of the derivative plots of the melting curve. The oligodeoxynucleotides were dissolved in a medium salt buffer (pH = 7.0, 1 mM EDTA, 10 mM Na₂HPO₄×2H₂O, 140 mM NaCl) to a concentration of 1.0 μM for each strand.

Example 27

Fluorescence when hybridized to mismatched targets

Quenching in fluorescence is a sign of strong interaction of the fluorophore with the duplex. Structural minimization calculations have supported that the pyrene moiety is intercalated into the duplex. It was therefore anticipated that the introduction of mismatches near the site of intercalation results in increased flexibility to the pyrene and hence increased fluorescence. This was also what was found irrespective to which side of the intercalator a mismatch is introduced (Table 6).

Table 6: Fluorescent data of mono modified ODN hybridised to either the complementary sequence or to one of six different neighbouring single point mutants.

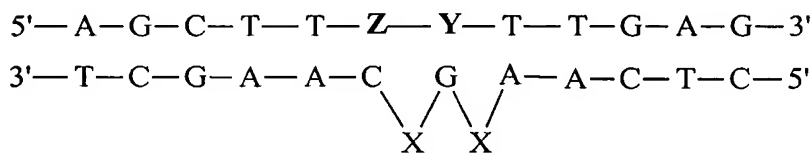


| Name | Z | Y | 382 nm Rel. In- tensity | 395 nm Rel. In- tensity | 480 nm. Rel. In- tensity |
|----------------|----|----|-------------------------------|-------------------------------|--------------------------------|
| Probe alone | -- | -- | 48 | 40 | 1 |
| Wt | G | C | 15 | 12 | 1 |
| Mut. 1 | C | C | 59 | 50 | 2 |
| Mut. 2 | A | C | 75 | 63 | 2 |

| | | | | | |
|--------|---|---|----|----|---|
| Mut. 3 | T | C | 50 | 42 | 2 |
| Mut. 4 | G | T | 34 | 29 | 2 |
| Mut. 6 | G | A | 63 | 53 | 2 |

To test the hypothesis that probe III could be used for detection of single point mutants, it was hybridised to target with all four variants of Y, and the intensity of the excimer band at 480 nm was significantly increased (Table 7) when a mismatch was introduced (Y = G, A, T). Again it was expected that the fluorescence of the bands at 382 and 395 nm would increase upon introduction of a mismatch, which was also observed. Surprisingly the fluorescence at 480 nm also increased with introduction of a mismatch at the 3' side of both intercalators (Z = C, A, T), indicating that the two pyrene moieties are able to interact with each other (Table 7). This would only be expected if a loop, large enough to let the pyrene moieties interact, is created. It is noteworthy that the fluorescence at 382 and 395 is quenched when hybridised to a complementary sequence, but increased when hybridizing to a sequence with one mismatch. It should therefore be possible to use all three wavelengths (382, 295 and 480) to differentiate between a fully complementary sequence and a complementary sequence with one mismatch.

Table 7: Fluorescent data of a double modified ODN hybridised to either the complementary sequence or to one of six different single point mutants.



| Name | Z | Y | 382 nm Rel. In- tensity | 395 nm Rel. In- tensity | 480 nm Rel. In- tensity |
|--------|----|----|-------------------------------|-------------------------------|-------------------------------|
| SsDNA | -- | -- | 44 | 38 | 17 |
| Wt | G | C | 19 | 17 | 4 |
| Mut. 1 | C | C | 84 | 73 | 14 |
| Mut. 2 | A | C | 74 | 64 | 10 |

| | | | | | |
|--------|---|---|----|----|------|
| Mut. 3 | T | C | 84 | 74 | 12.5 |
| Mut. 4 | G | T | 62 | 54 | 8 |
| Mut. 5 | G | G | 84 | 74 | 17 |
| Mut. 6 | G | A | 70 | 60 | 12 |

Example 28

Thermal denaturation studies.

Insertions of intercalators into DNA have been accompanied by decrease in specificity for hybridization to fully complementary sequences when compared to sequences with mismatches in the basepairs surrounding the intercalator. Experiments were aimed to test if this is also the case for duplexes with pyrene moiety insertions, placing mismatches to either side of the intercalator, next to and between two intercalators

The specificity is measured by the difference in the melting temperature between the fully complementary duplex and the duplex where one mismatch has been introduced. Melting temperature data are presented in Table 8.

Table 8: Melting temperature data for ODNs with different insertion patterns hybridised to either the complementary strand or one of the three possible point mutations at nucleotide #6 and #7. X=1.

| | |
|--|---------------|
| 5' A-G-C-T-T-Z-Y-T-T-G-A-G | Target |
| 3' T-C-G-A-A-C-G-A-A-C-T-C | I |
| 3' T-C-G-A-A-C $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ G-A-A-C-T-C | II |
| 3' T-C-G-A-A-C $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ G-A-A-C-T-C | III |
| 3' T-C-G-A-A-C $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ G-A-X-C-T-C | IV |
| 3' T-C-G-A $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ A-C-G-A $\begin{array}{c} \diagup \diagdown \\ \text{X} \end{array}$ A-C-T-C | V |

| Tar- | | Probe | | | | | | | | | | |
|--------|---|-------|------|-------|------|-------|------|-------|------|-------|------|-------|
| get | | | | | | | | | | | | |
| Z | Y | I | II | | III | | IV | | V | | | |
| | | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] | [°C] |
| Wt | G | C | 47.4 | ΔT | 50.4 | ΔT | 51.4 | ΔT | 45.4 | ΔT | 60.8 | ΔT |
| Mut. 1 | C | C | 23.4 | -24.0 | 34.0 | -16.0 | 38.0 | -13.4 | 23.4 | -22.0 | 33.8 | -27.0 |
| Mut. 2 | A | C | 30.8 | -16.6 | 34.2 | -16.2 | 36.6 | -14.8 | | | | |
| Mut. 3 | T | C | 27.6 | -18.8 | 33.6 | -16.8 | 35.2 | -16.2 | 25.4 | -20.0 | 37.4 | -23.4 |
| Mut. 4 | G | T | 36.2 | -11.2 | 42.2 | -8.2 | 45.2 | -6.2 | 36.6 | -8.8 | 45.8 | -15.0 |
| Mut. 5 | G | G | 40.0 | -7.4 | 42.4 | -8.0 | 38.6 | -12.8 | 39.4 | -6.0 | 53.2 | -7.6 |
| Mut. 6 | G | A | 39.8 | -7.6 | 39.0 | -11.4 | 39.0 | -12.4 | 39.2 | -6.2 | 49.0 | -11.8 |

As seen from Table 8 the specificity against mismatches of the modified probe is in the range of that of the unmodified probe, though there seems to be a drop in selectivity for C-C mismatches to the 5' end of the intercalation site (Table 8; Mut. 1 with Probe II and III). The only other consistent trend is that the double modified probe where the two intercalators are separated by four nucleotides (probe V) is more specific against mismatches two nucleotides away from any of the intercalation sites than the unmodified probe (probe I). The rest of the melting temperature differences are close to the values for the unmodified duplexes. Most important in respect to the search for single point mutants is that probe III is selective for its target having the right base in between the pyrene moieties, which is the case and in two out of the three possible mismatches it is even more specific, being less specific in the last instance (Table 8).

Example 29

Beacon-primers

An example of a Beacon-design primer is given in figure 40. The primer consists of 39 nucleotides, which are designed so that they can form a stem-loop structure.

The primer has a target-complementary region, that is complementary to the target DNA, which is 24 nucleotides long. Furthermore, the primer has a self-complementary region, that is capable of hybridising to the other end of the primer. The self-complementary region is 15 nucleotides long and comprises furthermore 4 intercalator pseudonucleotides. Two of the intercalator pseudonucleotides are positioned so they are capable of forming an intramolecular excimer.

The melting temperature of the primer/target hybrid is around 67°C, whereas the melting temperature of the selfhybrid is around 46°C.

The beacon primer can be used for PCR and allows quantification of the PCR.

Target specific beacon primers and template DNA is provided (figure 41A). The beacon primers and the template DNA is stored at 0-4°C.

Double stranded DNA is denatured at 94°C (figure 41B). The beacon primers are annealed to the target DNA at around 65°C (figure 41C) and the primers are elongated by Taq polymerase.

Subsequently, the temperature is lowered to around 45°C and unhybridised beacon primers are hybridised to itself (figure 41D). Excimer fluorescence is determined and correlated to the amount of elongated beacon primer.

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Claims

1. An oligonucleotide or oligonucleotide analogue comprising at least two
 5 intercalator pseudonucleotides of the general structure



wherein

10

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue; and

15

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator,

20

and wherein the at least two intercalator pseudonucleotides are separated by n nucleotides, wherein n is selected from the group consisting of integers in the range from 1 to 12.

25

2. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the oligonucleotide or oligonucleotide comprises one or more selected from the group consisting of subunits of DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, α -L-RNA, α -D-RNA, β -D-RNA.

30

3. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators of 2 intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer

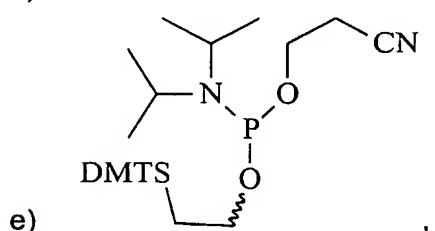
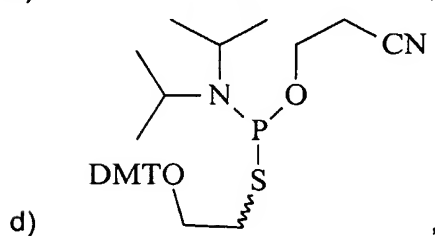
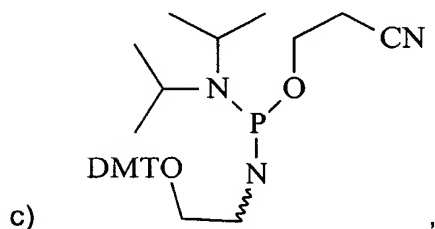
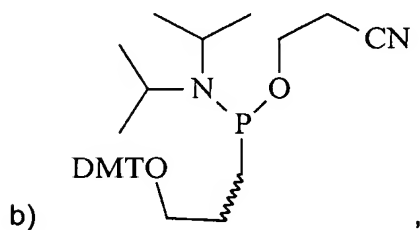
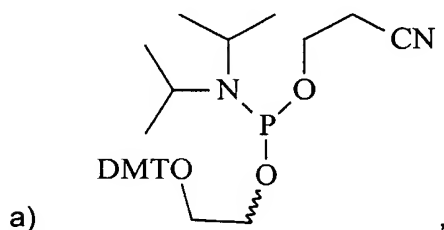
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complex, when at least one of the n nucleotides separating the intercalator pseudonucleotides is unhybridised.

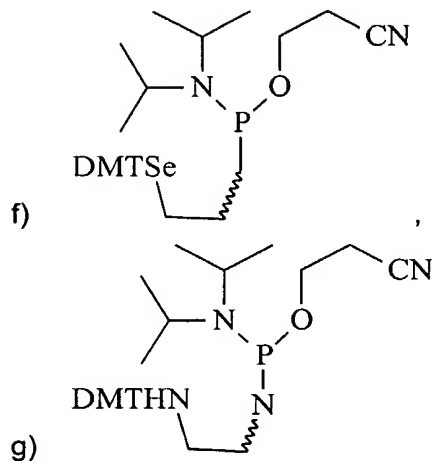
- 5 4. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators of 2 intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex, when the n nucleotides separating the intercalator pseudonucleotides are unhybridised and wherein the intercalators are not capable of forming an intramolecular excimer, when the n nucleotides separating the intercalator
10 pseudonucleotides are hybridised to complementary nucleotides.
5. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein n is 1.
- 15 6. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the oligonucleotide comprises exactly 2 intercalator pseudonucleotides.
7. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the at least 2 intercalator pseudonucleotides are similar.
20
8. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the at least 2 intercalator pseudonucleotides are different.
9. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein
25 the oligonucleotide or oligonucleotide analogue consists of between 5 and 100 nucleotides and/or intercalator pseudonucleotides.
10. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the backbone monomer unit is selected from the group consisting of acyclic
30 backbone monomer units.
11. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the backbone monomer unit is selected from the group consisting of acyclic backbone monomer units capable of stabilising a bulge insertion.
35

12. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit is selected from the group consisting of phosphoramidites.

5 13. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the backbone monomer units are selected from the group consisting of backbone monomer units with the following structures



223



14. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein
5 the intercalators comprise a chemical group selected from the group consisting of polyaromates and heteropolyaromates.
15. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein
10 the intercalators are selected from the group consisting of polyaromates and heteropolyaromates.
16. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein
15 the intercalators are selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.
17. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at
20 least one intercalator is pyrene.
18. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein
the linkers comprise a chain of m atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb.
- 25 19. The oligonucleotide or oligonucleotide analogue according to claim 18, wherein m is selected from the group of integers from 1 to 7, preferably from 2 to 5, most preferably 3.

20. The oligonucleotide or oligonucleotide analogue according to claim 18, wherein the chain is substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.
- 5
21. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is an alkyl chain.
22. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.
- 10
23. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb.
- 15
24. The oligonucleotide or oligonucleotide analogue according to claim 23, wherein the linker is substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.
- 20
25. The oligonucleotide or oligonucleotide analogue according claim 1, wherein at least one intercalator pseudonucleotide is selected from the group consisting of 1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol.
- 25
26. The oligonucleotide or oligonucleotide analogue according claim 1, wherein at least one intercalator pseudonucleotide is selected from the group consisting of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol and (R)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol.
- 30
27. The oligonucleotide analogue according to claim 1, wherein the intercalators of at least 2 intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex.

225

28. The oligonucleotide analogues according to any of claim 27, wherein a fluorescent signal is changed upon hybridisation to a corresponding nucleic acid or nucleic acid analogue.
- 5 29. A method of detecting a nucleic acid or nucleic acid analogue comprising a specific target sequence, which may differ from any other sequences by at least one nucleobase, wherein the method comprises the steps of
- 10 a) providing a mixture comprising nucleic acids and/ or nucleic acid analogues, which is desirable to test for the presence of the target sequence; and
- b) providing an oligonucleotide analogue comprising at least one intercalator pseudonucleotide capable of hybridising with said specific sequence; and
- c) incubating the oligonucleotide analogue with the mixture comprising nucleic acids or nucleic acid analogues under conditions allowing for hybridisation;
- 15 and
- d) washing away sequences that have less affinity to said oligonucleotide analogue than the target sequence; and
- e) determining the presence or absence the target sequence.
- 20 30. A method of differentiating between a nucleic acid or nucleic acid analogue comprising a specific target sequence and a nucleic acid comprising a mutant sequence which differs from the target sequence by at least one nucleobase, wherein the method comprises the steps of
- 25 a) providing a mixture comprising nucleic acids or nucleic acid analogues, which is desirable to test for the presence of a target sequence or a mutant sequence; and
- b) providing an oligonucleotide analogue comprising at least one intercalator pseudonucleotide capable of hybridising with said target sequence; and
- 30 c) incubating said oligonucleotide analogue with the mixture comprising nucleic acids or nucleic acid analogues under conditions allowing for hybridization; and
- d) determining the presence or absence of said target sequence and/or the presence of the mutant sequence.

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226

31. The method according to any of claims 29 and 30, wherein the intercalator pseudonucleotide is intercalator pseudo nucleotide of the general structure



5

wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,

10

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of DNA; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

15

wherein the total length of Q and Y is in the range from 8 Å to 13 Å,

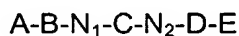
with the proviso that when the intercalator is pyrene the total length of Q and Y is in the range from 9 Å to 11 Å.

20

32. The method according to any of claims 29 and 30, wherein the oligonucleotide analogue is the oligonucleotide analogue according to any of claims 1 to 28.

25

33. The method according to any of claims 29 to 32, wherein the oligonucleotide analogue is of the structure



30

wherein A is a sequence essentially complementary to one sequence directly flanking the potential mutation of the mutant sequence; and

E is a sequence essentially complementary to the other sequence directly flanking the potential mutation of the mutant sequence; and

35

227

N_1 and N_2 are intercalator pseudonucleotides, which may or may not be identical; and

5 C is a sequence complementary to the target sequence but not complementary to the mutant sequence or C is a sequence complementary to the mutant sequence but not the target sequence; and

10 B is a sequence complementary to the target sequence but not complementary to the mutant sequence or B is a sequence complementary to the mutant sequence but not the target sequence; and

15 D is a sequence complementary to the target sequence but not complementary to the mutant sequence or D is a sequence complementary to the mutant sequence but not the target sequence.

34. The method according to any of claims 29 to 32, wherein the presence or absence of the mutation is determined by determining the spectral properties of the oligonucleotide or oligonucleotide analogue after hybridisation.

20 35. The method according to claim 34, wherein the spectral properties are fluorescence properties.

25 36. The method according to claim 34, wherein the spectral properties are selected from the group consisting of monomer fluorescence, excimer fluorescence, exciplex fluorescence, FRET and charge transfer fluorescence.

37. The method according to claim 34, wherein the spectral properties are monomer fluorescence and excimer or exciplex or FRET or charged transfer fluorescence.

30 38. The method according to claim 36, wherein low monomer fluorescence is indicative of hybridisation with the specific target sequence.

39. The method according to any of claims 29 to 32, wherein the presence or absence of the mutant sequence is determined by determining the melting

228

temperature of a hybrid between the oligonucleotide or oligonucleotide analogue and the test nucleic acid or nucleic acid analogue.

- 5 40. The method according to claim 39, wherein a high melting temperature is indicative of hybridisation to the mutant sequence and a low melting temperature is indicative of hybridisation to the target sequence.
- 10 41. The method according to claim 39, wherein a high melting temperature is indicative of hybridisation to the target sequence and a low melting temperature is indicative of hybridisation to the mutant sequence.
- 15 42. The method according to any of the claims 30 to 32, wherein the mutant sequence comprises a polymorphism.
- 20 43. The method according to claim 42, wherein the polymorphism is indicative of a specific DNA profile.
44. The method according to claim 43, wherein the specific DNA profile is employed to identify an individual.
45. The method according to claim 43, wherein the specific DNA profile is employed to determine relationship between individuals.
- 25 46. The method according to any of the claims 30 to 32, wherein the mutant sequence comprises a point mutation compared to the target sequence.
47. The method according to any of the claims 30 to 32, wherein the presence of the mutant sequence is indicative of a clinical condition.
- 30 48. The method according to any of the claims 30 to 32, wherein the presence of the mutant sequence is indicative of increased risk of a clinical condition.
- 35 49. The method according to any of claims 47 to 48, wherein the clinical condition is selected from the group consisting of neoplastic diseases, neurodegenerative diseases, cardiovascular diseases and metabolic disorders including diabetes.

50. The method according to any of the claims 30 to 32, wherein the presence of the mutant sequence is indicative of a specific response to a predetermined drug treatment.
- 5
51. The method according to any of the claims 29 to 32, wherein the mixture comprising nucleic acid sample is selected from the group consisting of genomic DNA and an amplification product of genomic DNA.
- 10
52. The method according to any of claims 29 to 32, wherein hybridization is carried out under high stringency conditions.
53. The method according to any of claims 29 to 32, wherein hybridization is followed by a separation step separating all non hybridised nucleic acids or
- 15
- nucleic acid analogues from hybridised nucleic acid or nucleic acid analogues.
54. The method according to any of claims 29 to 32, wherein said oligonucleotide analogue is attached to at least one label, which is not a part of an intercalator pseudonucleotide.
- 20
55. A method for detecting a target sequence and/or a mutant sequence, which differ from the target sequence by at least one nucleobase, said method comprising the steps of
- 25
- a) providing a mixture comprising nucleic acids and/ or nucleic acid analogues, which are desirable to test for the presence of the target sequences or the mutant sequence; and
- b) providing at least one oligonucleotide analogue comprising at least one intercalator pseudonucleotide, wherein said oligonucleotide analogue is
- 30
- capable of hybridizing with said target sequence and/or the mutant sequence; and
- c) incubating said oligonucleotide analogue with said mixture of nucleic acids and/ or nucleic acid analogues under conditions allowing for hybridization; and

230

- d) using said hybridized target sequence for templating extension of the 3' end of said oligonucleotide analogue with a label or labeled nucleotide or labeled nucleotide analogue or labeled oligonucleotide or labeled oligonucleotide analogue; and
- 5 e) optionally separating hybridized and unhybridized sequences; and
- f) determining the presence or absence of the target sequence and/or mutant sequence.
- 10 56. The method according to claim 55, wherein extension is carried out enzymatically.
57. The method according to claim 55, wherein extension is carried out by a DNA polymerase.
- 15 58. The method according to claim 55, wherein extension is carried out by a ligase.
59. The method according to claim 55, wherein the method is used for detection of gene expression.
- 20 60. The method according to claim 55, wherein the method is used for differentiating between target nucleic acid sequences and mutant sequences.
61. The method according to claim 55, wherein the method is used for genotyping of single nucleotide polymorphisms.
- 25 62. The method according to claim 55, wherein the extension is carried out in the presence of more than one label, labeled nucleotide, labeled nucleotide analogue, labeled oligonucleotide or labeled oligonucleotide analogue.
- 30 63. The method according to claim 62, wherein the labels are distinguishable from each other.
64. The method according to any of claims 29 to 32 or 55, wherein at least one oligonucleotide analogue is affixed on a solid support.
- 35

231

65. The method according to any of claims 29 to 32 or 55, wherein more than one kind of oligonucleotide analogues are provided which comprises different sequences and wherein the different oligonucleotide analogues are affixed to a solid support in individual areas.

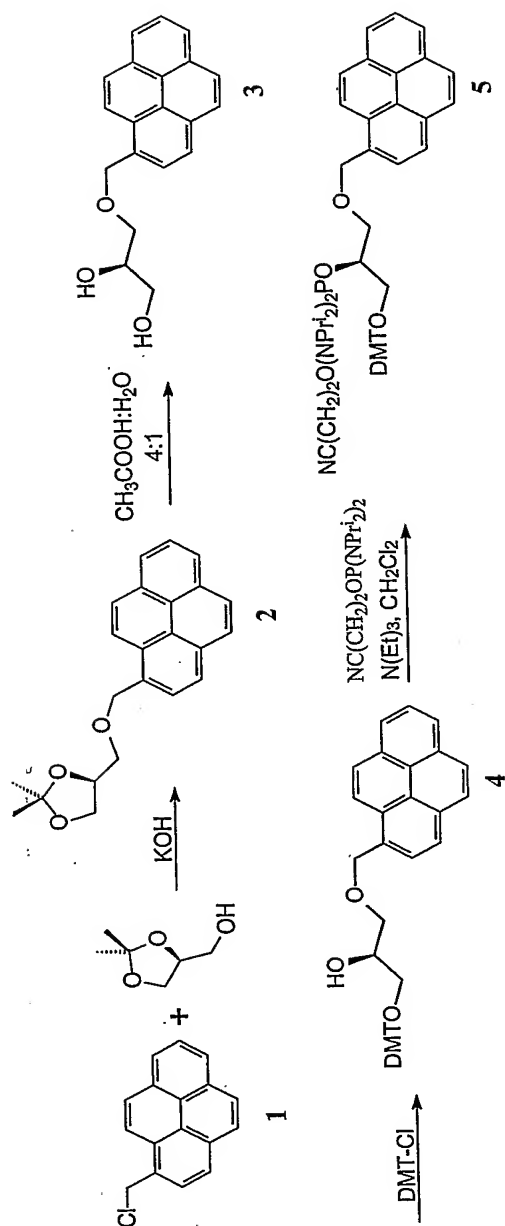
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66. The method according to any of claims 64 to 65, wherein the solid support is selected from the group consisting of chip array supports and microtiter plates.

10

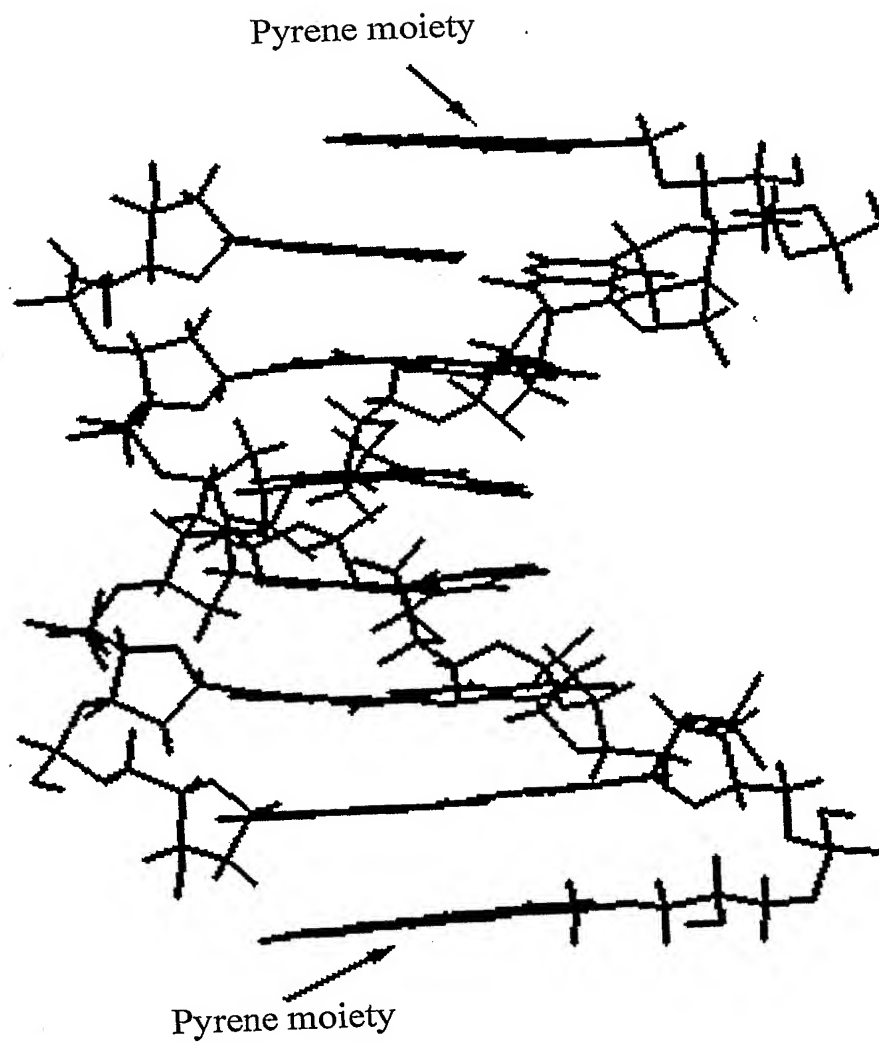
1/32

Fig. 1



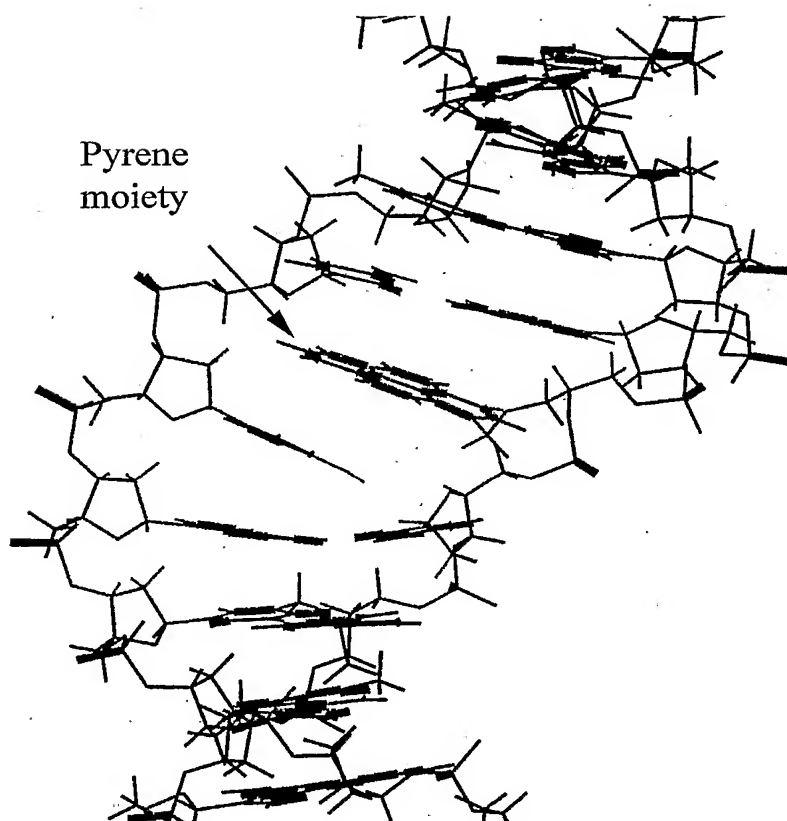
2/32

Fig. 2



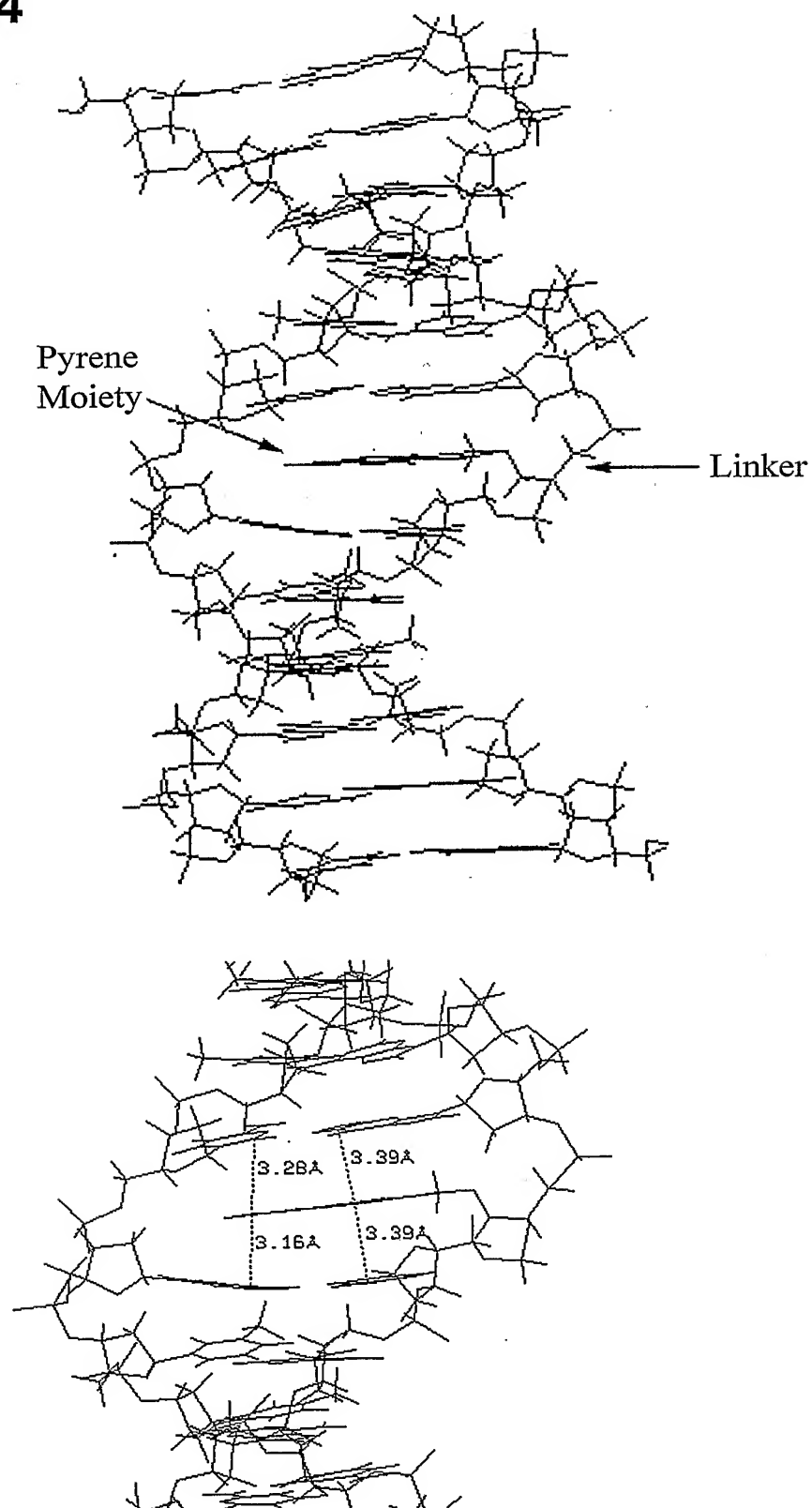
3/32

Fig. 3



4/32

Fig. 4



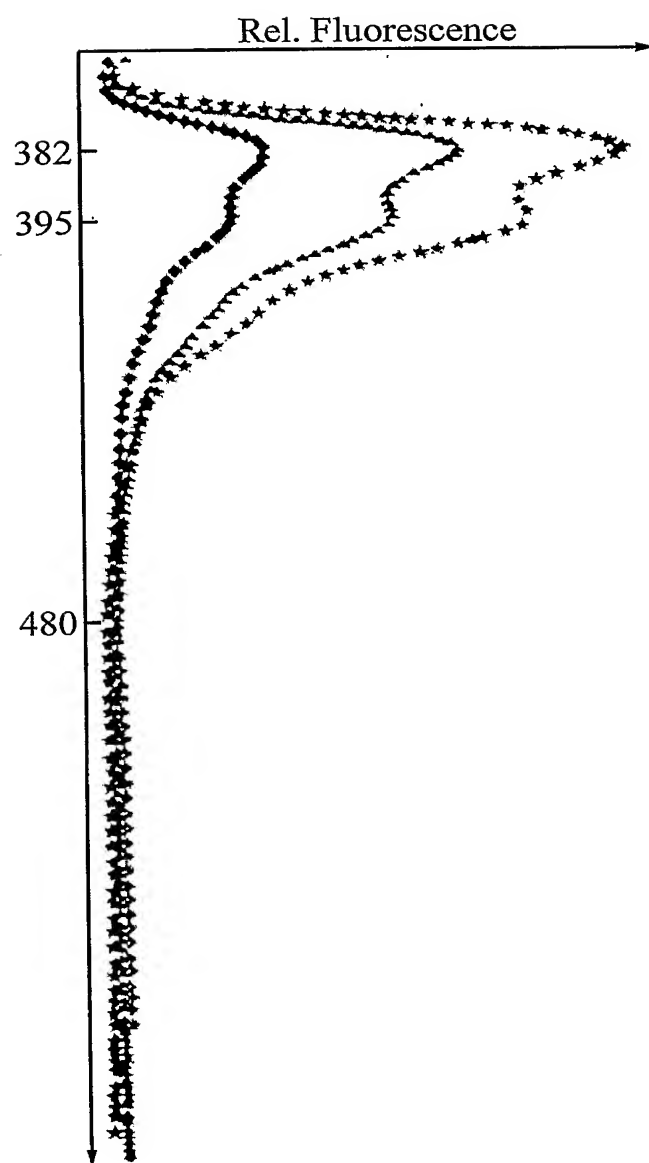
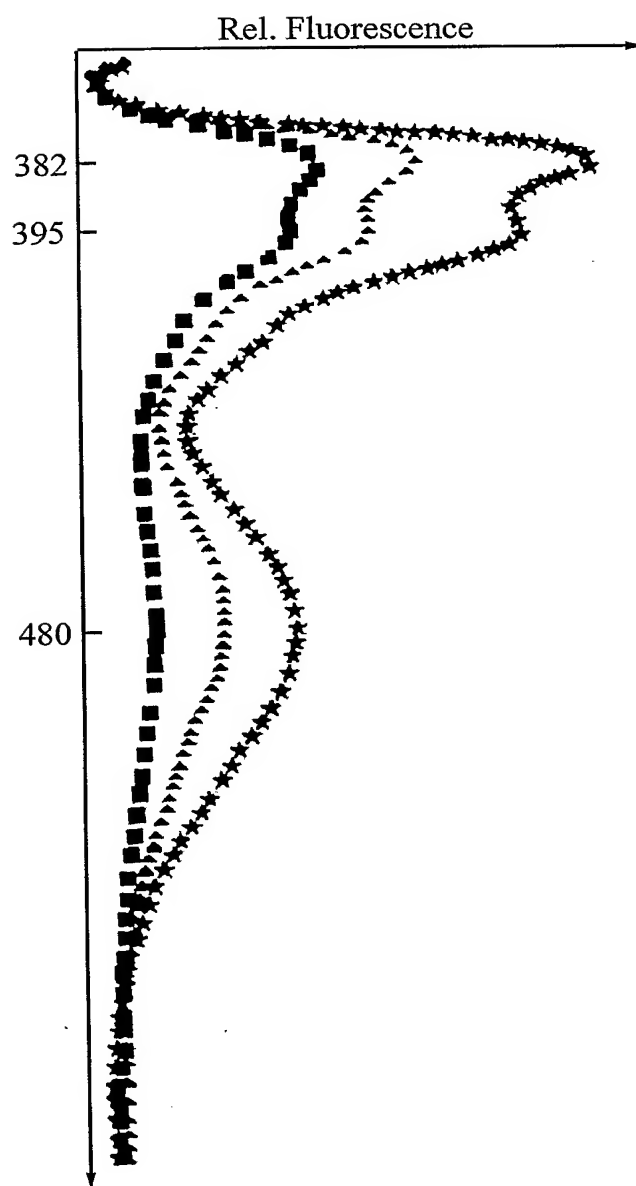
5/32**Fig. 5**

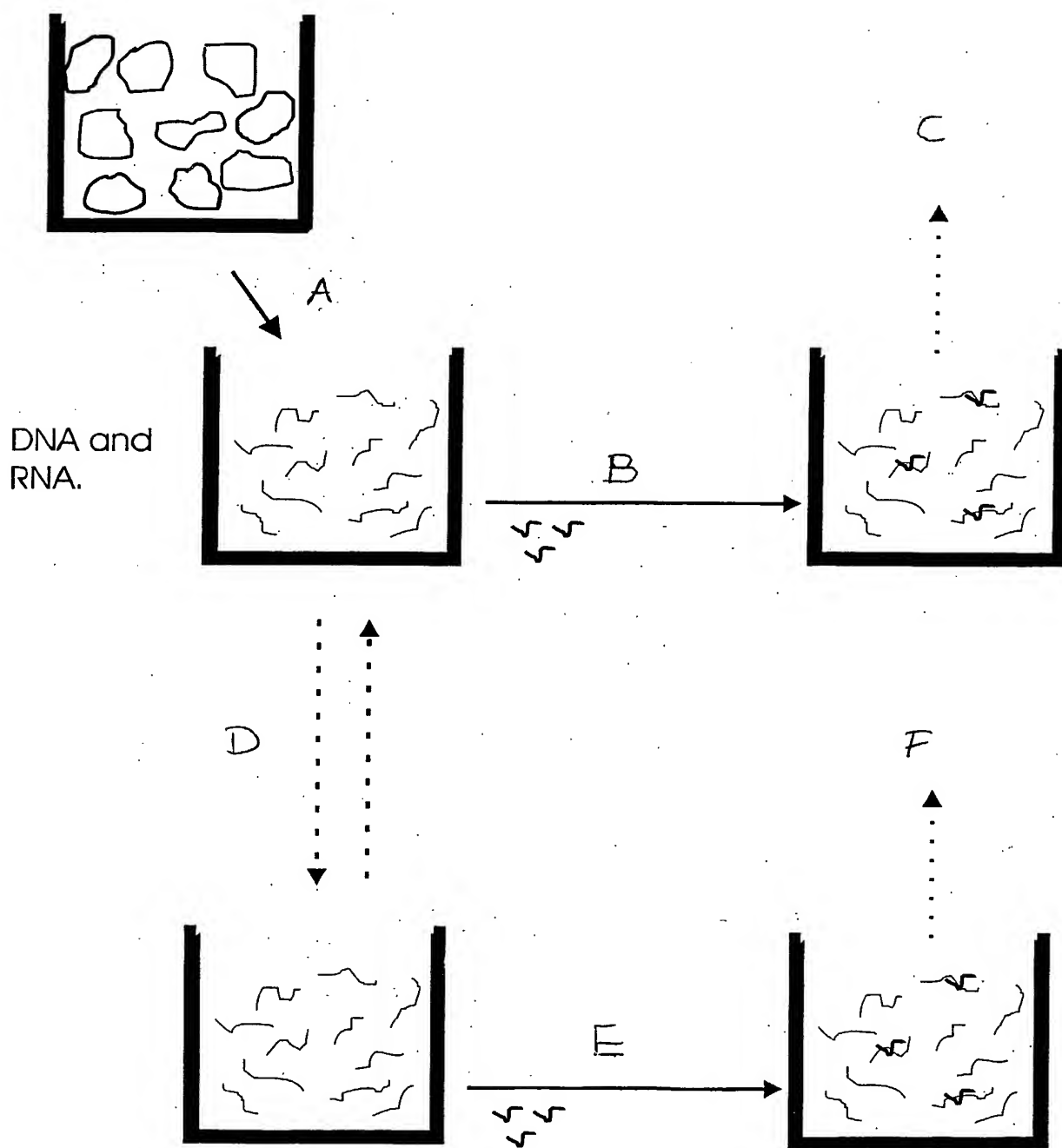
Fig. 6

6/32



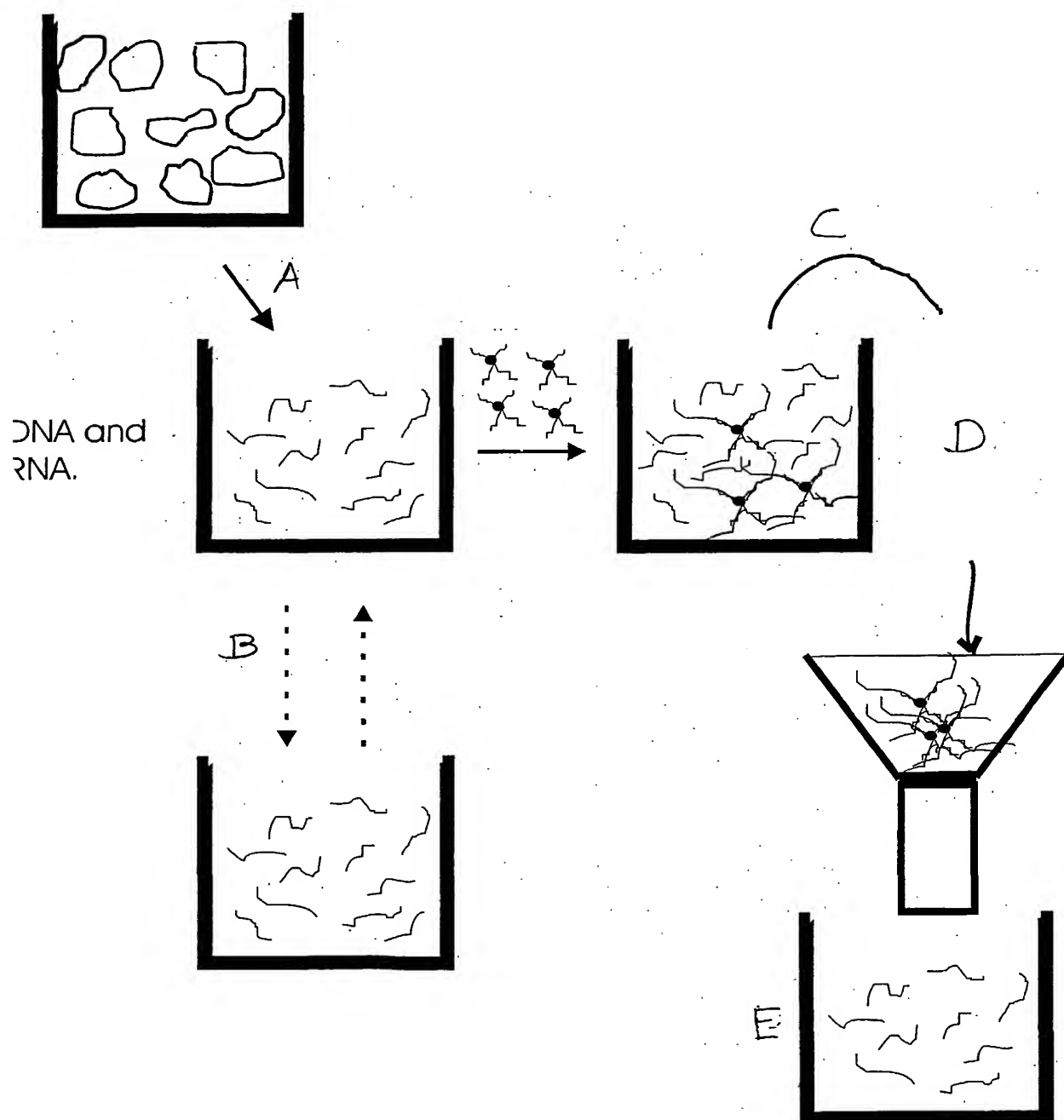
7/32

Fig. 7



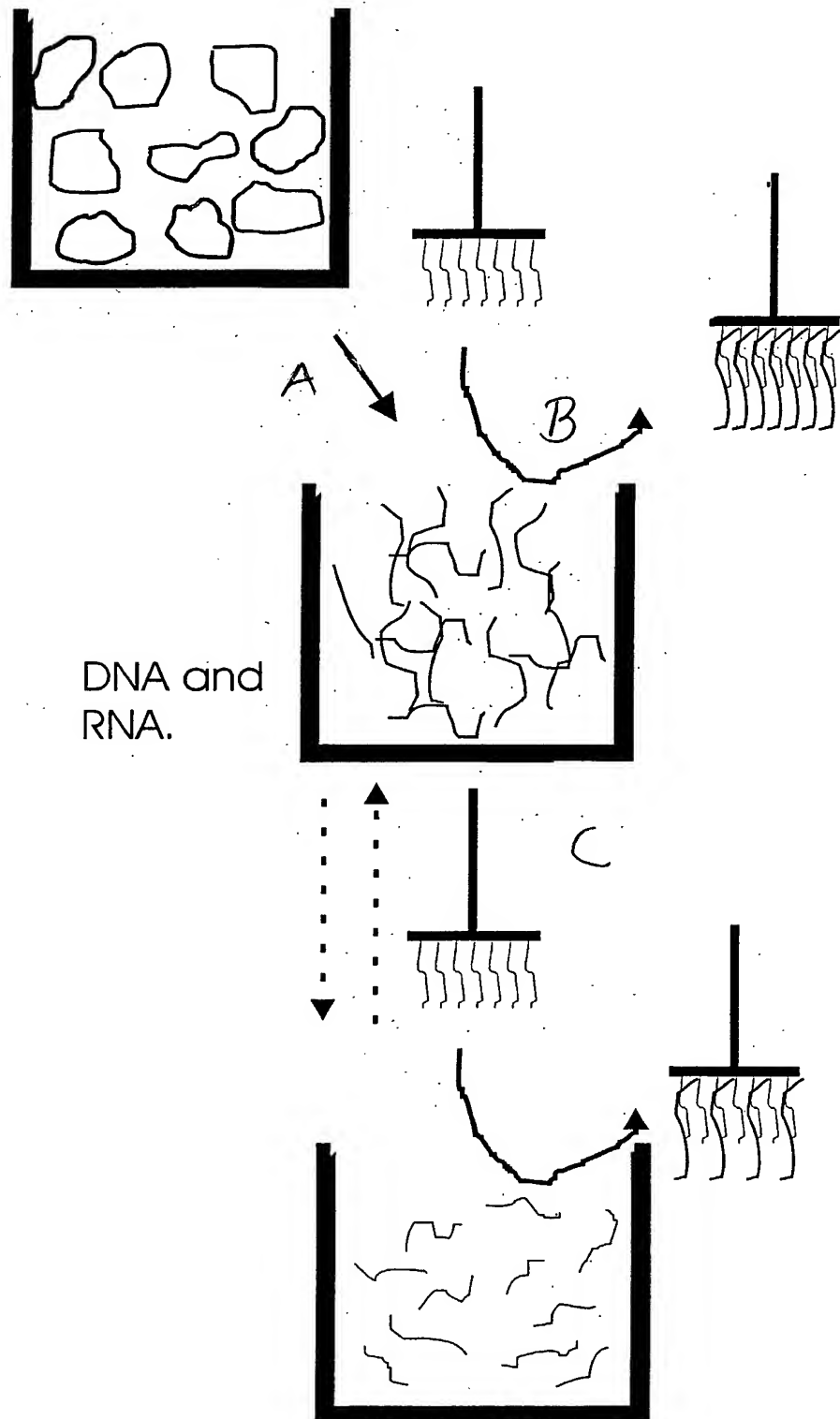
8/32

Fig. 8



9/32

Fig. 9



10/32

Fig. 10

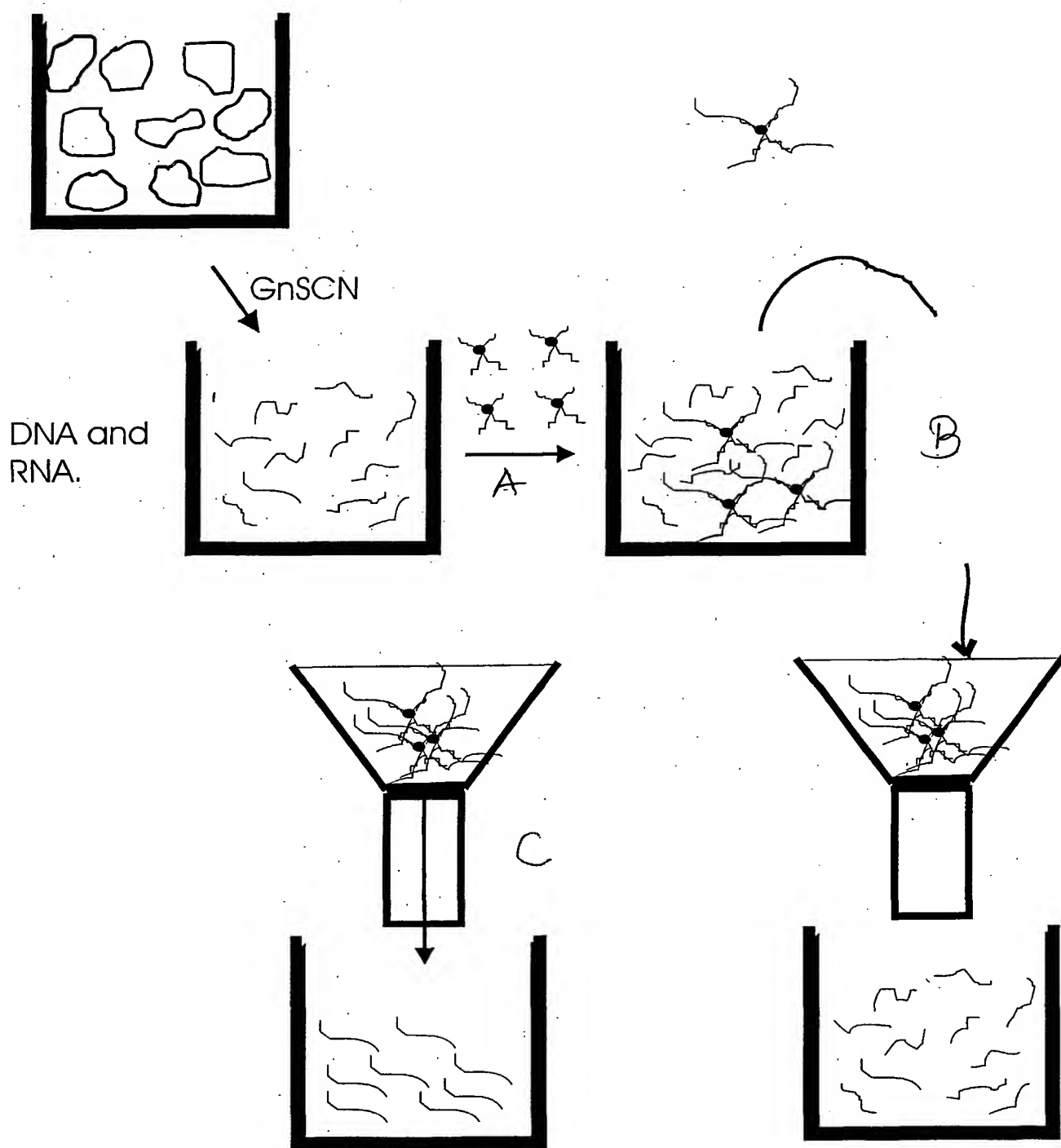


Fig. 11

11/32

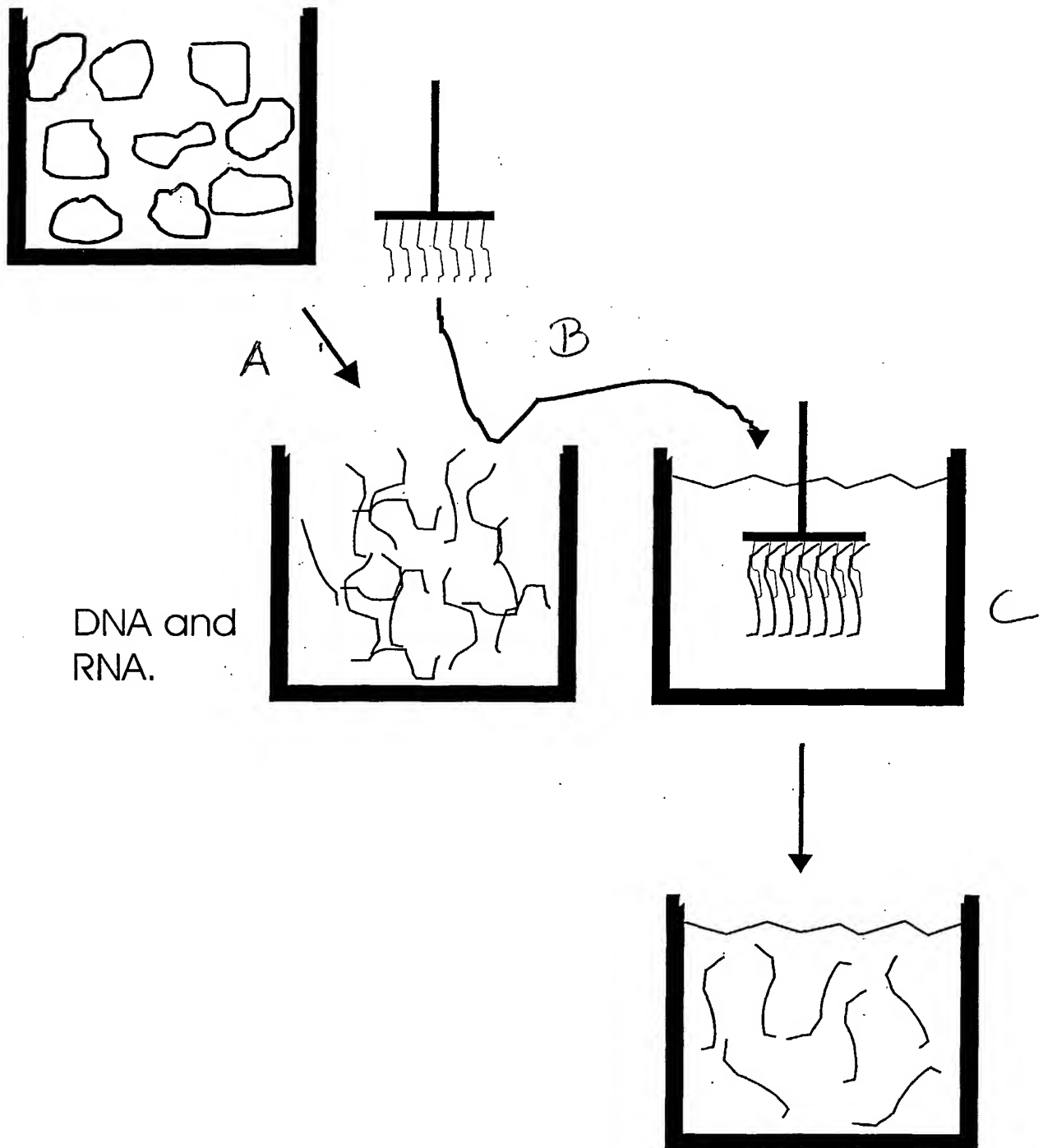
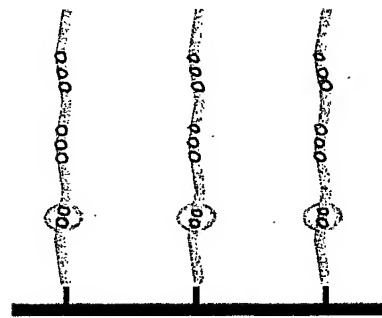
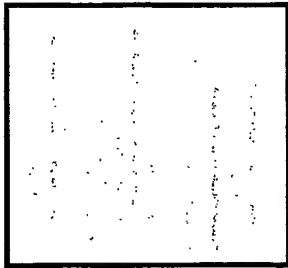


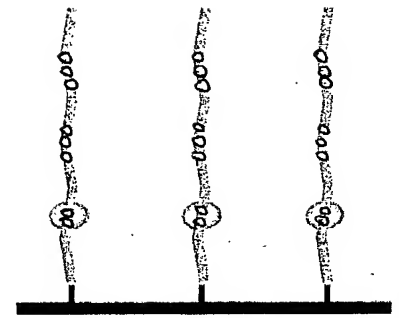
Fig. 12

12/32

1

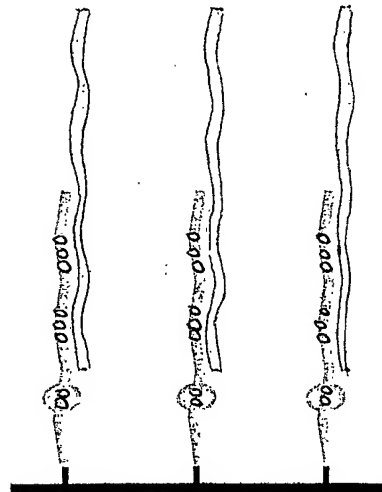


Spot 1

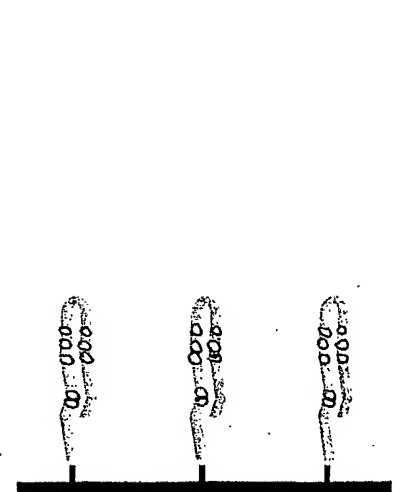


Spot 2

2



Spot 1



Spot 2

13/32

Fig. 13

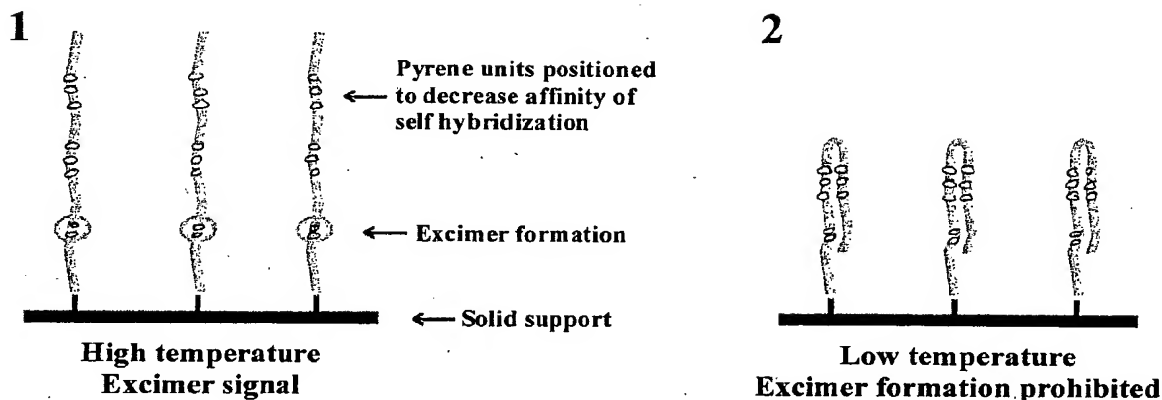
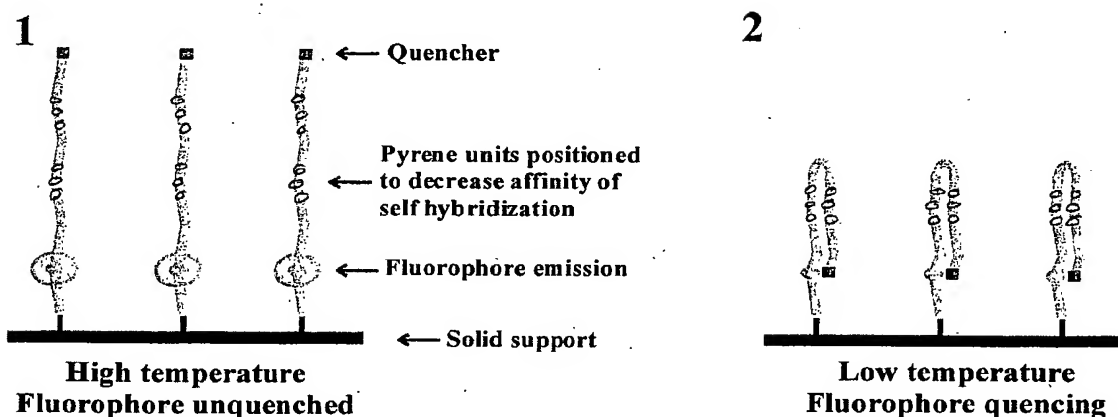
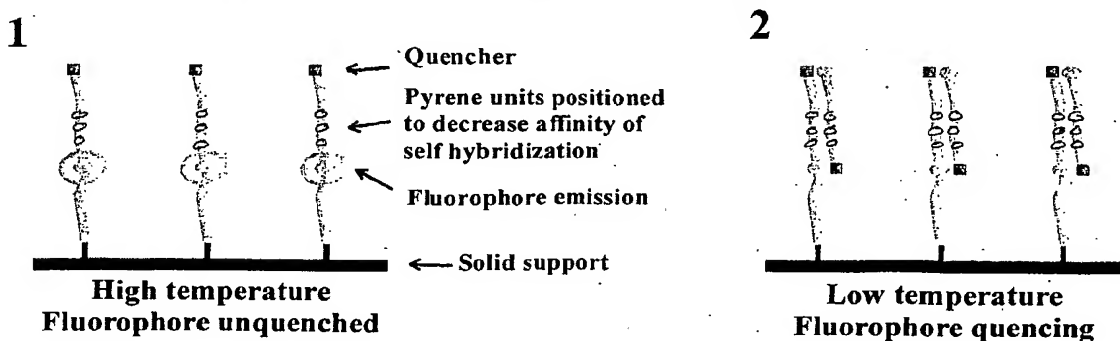
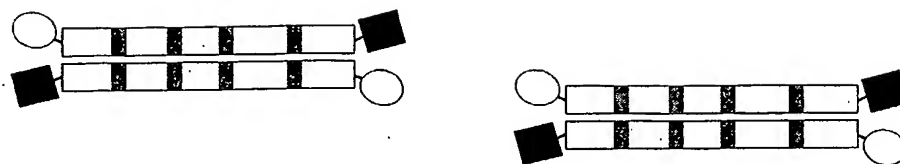
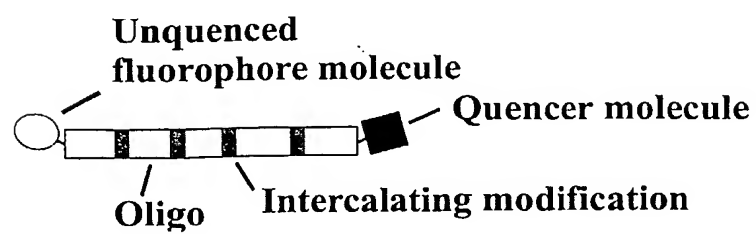
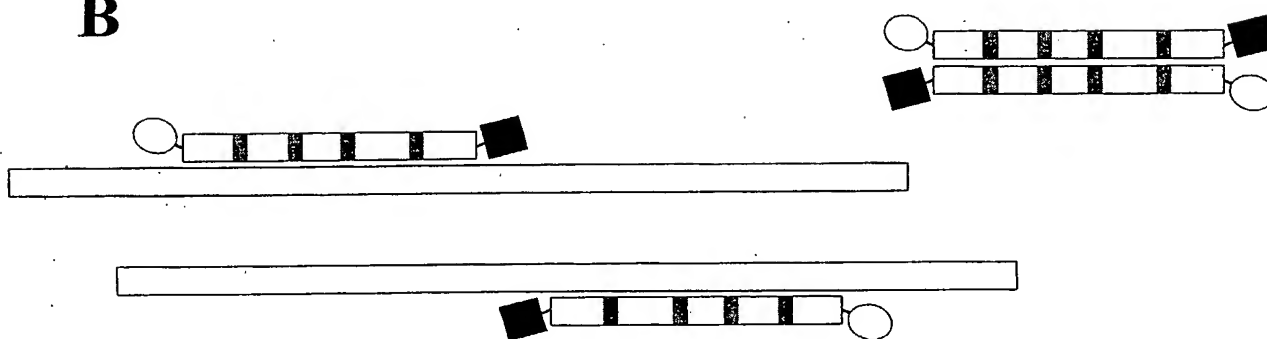
Basal Chip states**A Excimer Chip****B Traditional Fluorophore Chip****C Beacon Sandwich Fluorophore Chip**

Fig. 14

14/32

A**B**

15/32

Fig. 15

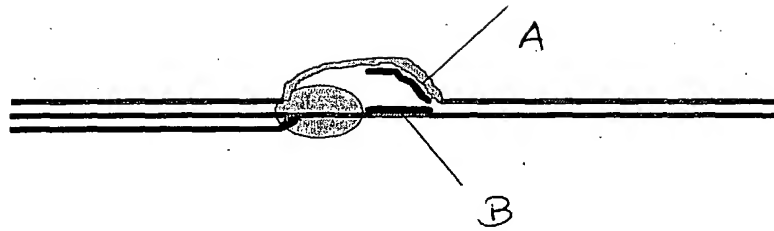


Fig. 16

16/32

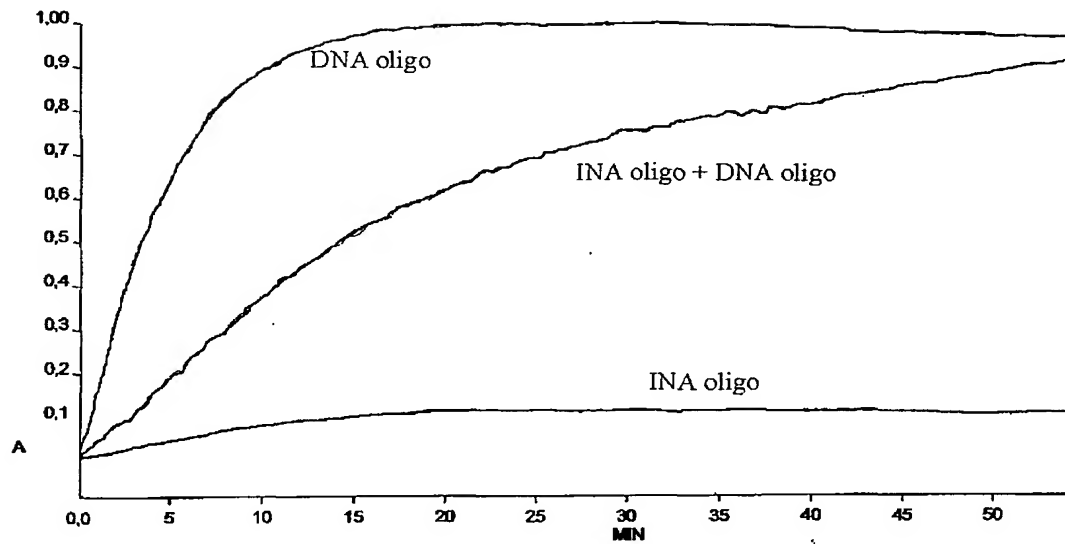
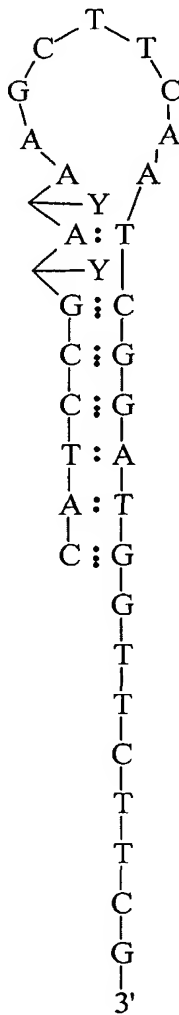


Fig. 17



17/32

Fig. 18

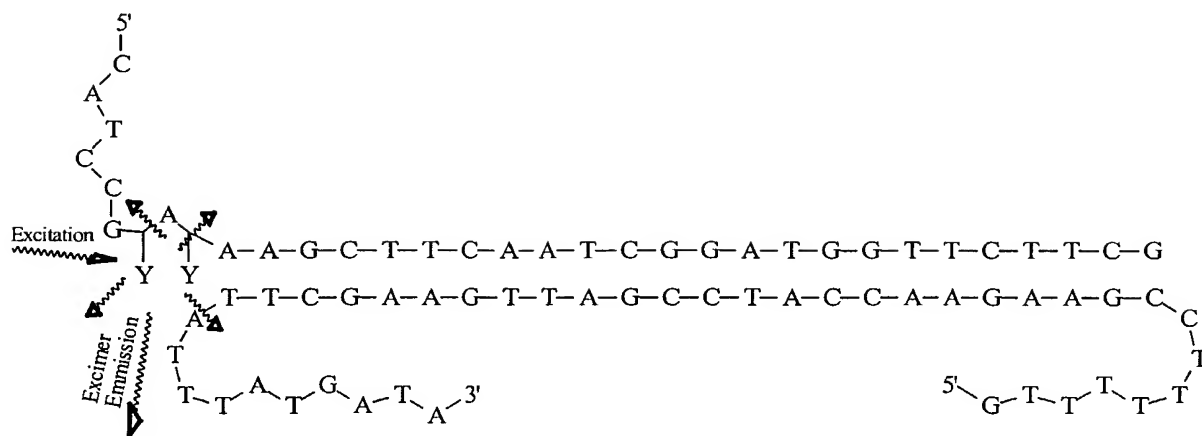
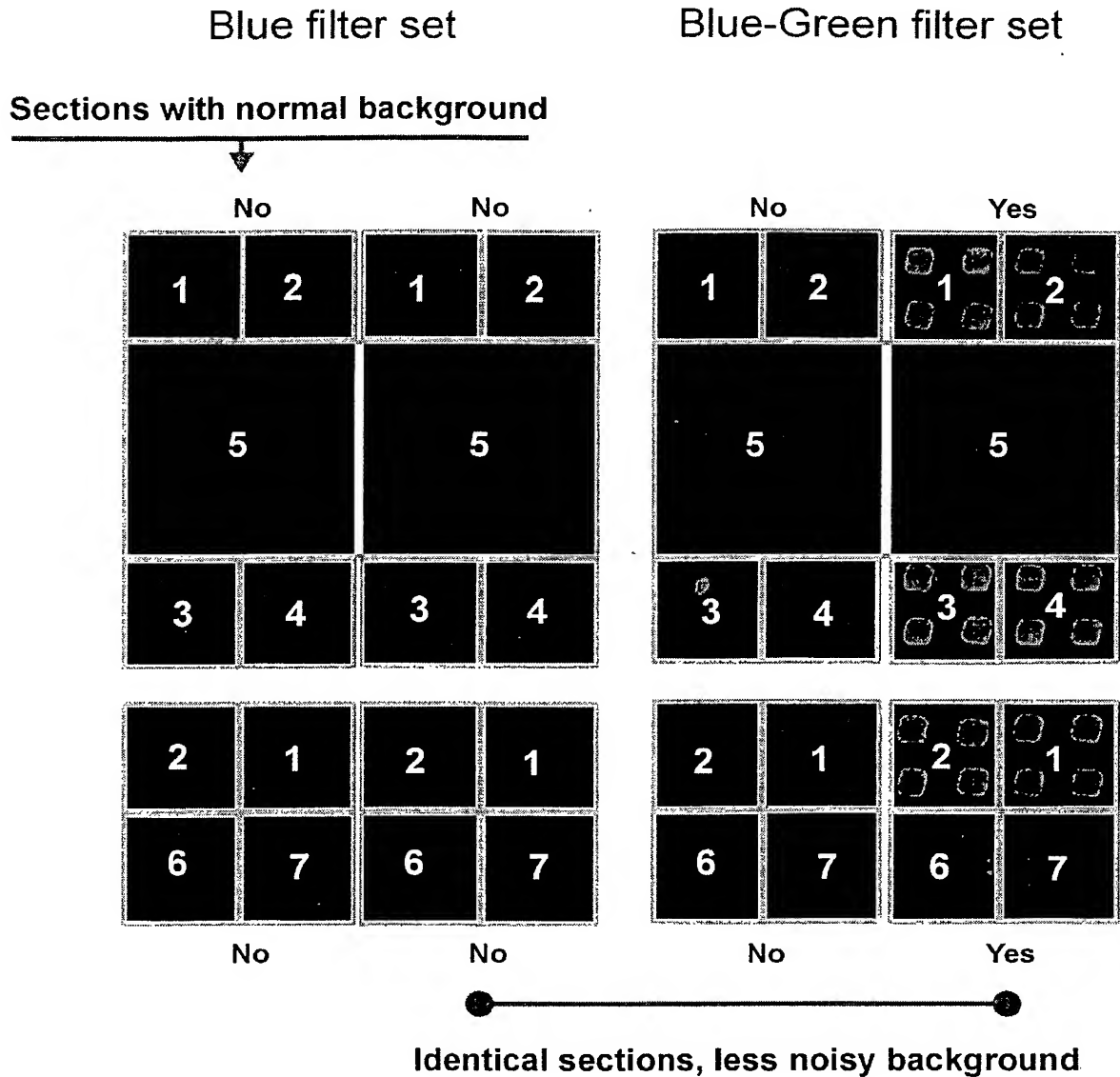


Fig. 19



Fig. 20

18/32

Testing oligo binding on Asper SAL slides**Labels**

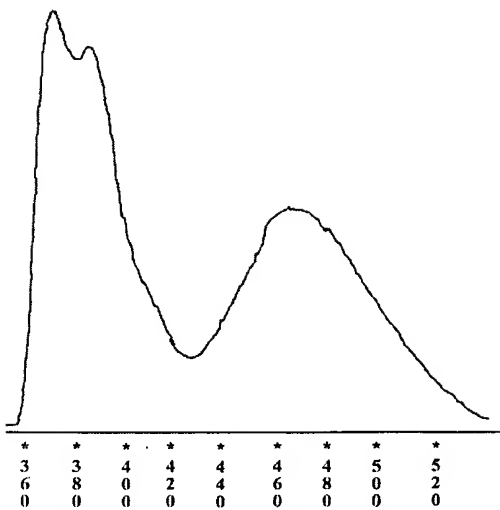
INA-oligos with linker & signal (1, 2, 3 & 4)

Short INA oligos, no linker or signal (5)

Buffer only (6), normal oligo with linker (7)

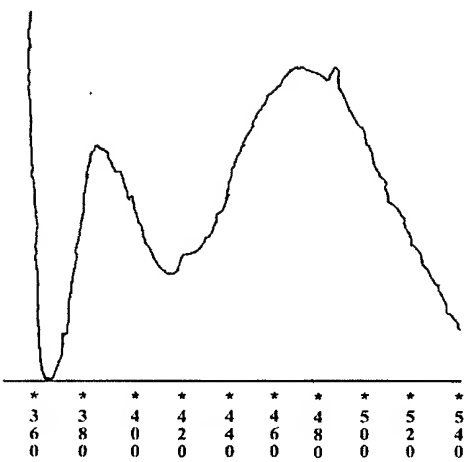
SYBR Green (Yes/ No)

Fig. 21



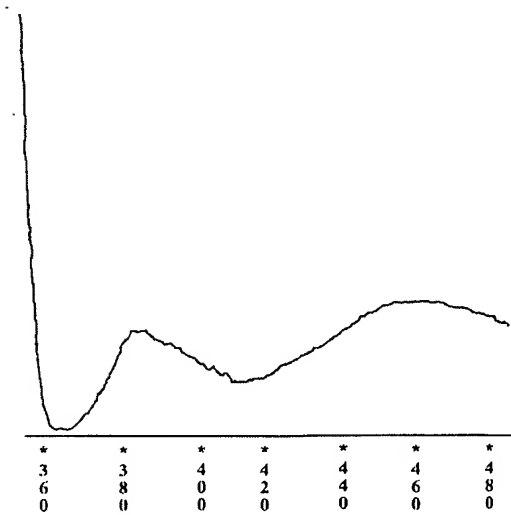
Exciplex between molecules X and Y when placed as next-nearest neighbors (sequence I)

Fig. 22



Exciplex fluorescence between the to molecules X and Y when placed as neighbours (sequence II)

Fig. 23



Exciplex fluorescence between molecules Y and Z when placed as neighbours

Fig. 24

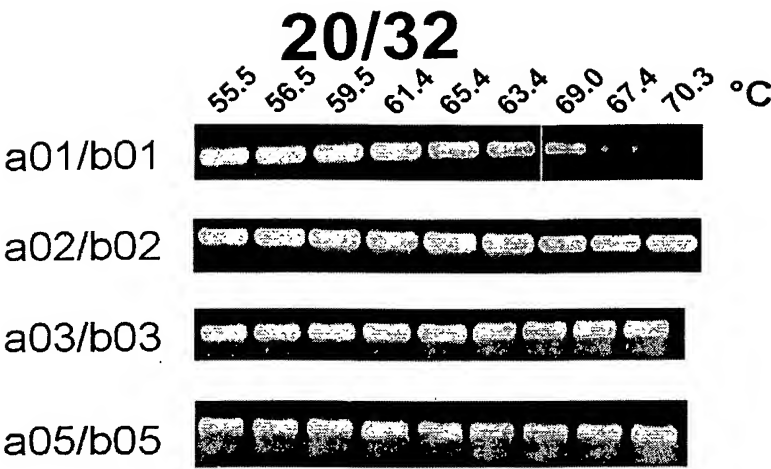


Fig. 25

Target 5'- GGGCTTTAAAGCCTCGCGGGGCTGACAGGTGAAATCGGCGCGGAAGCTGTCGGGGTAGCGTCGACGCGCCTAGGGGAT

Target 3'- CCCGAAATTTCGGAGCGCCCGGACTGTCCACTTTAGCCGCGCCTTCGACAGCCCATCGCAGACGTGCGGGATGCCCTA

IO 1-1: 3'- ACTYTTAGYCCGYCGCCTTCGYA

IO 1-2: 3'- ACTYTTAGYCCGYCGCCTTCYGA

IO 1-3: 3'- ACTYTTAGYCCGYCGCCTTYCGA

IO 1: 5'- TGAAATCGGCGCGGAAGCTYG

IO 3: 5'- TGAAATCGGCGCGGAYAGCTYG

IO 5: 5'- TGAYAATCYGGCYGCGGAYAGCTYG

Fig. 26

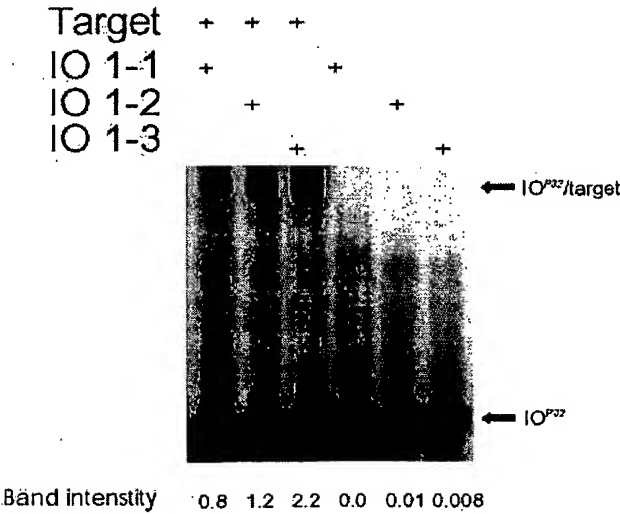


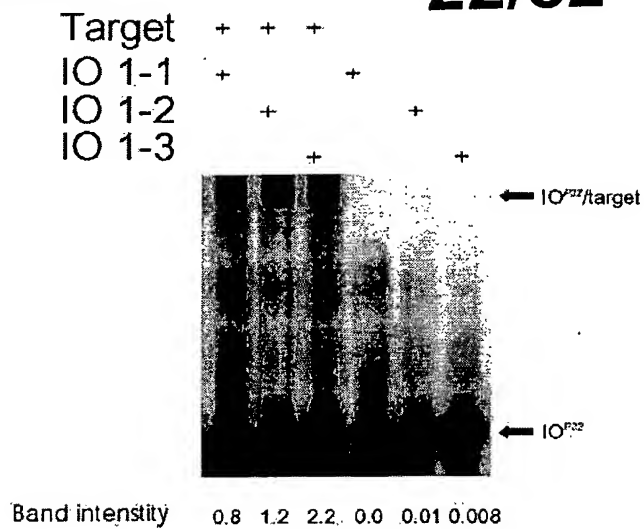
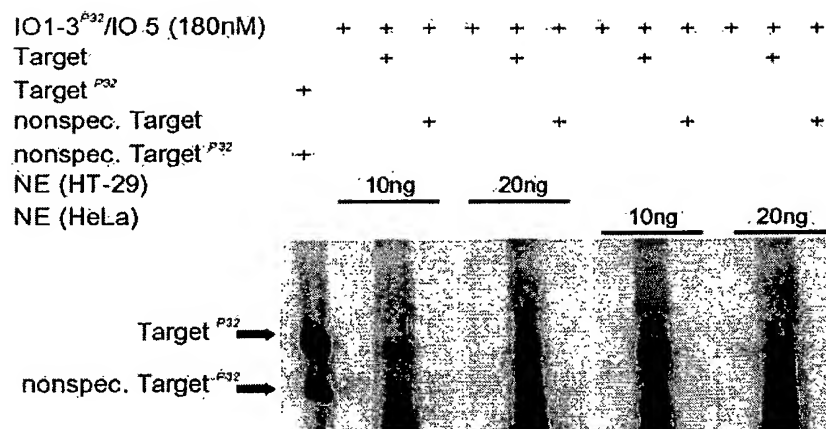
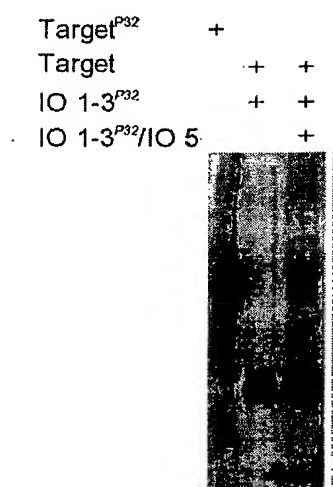
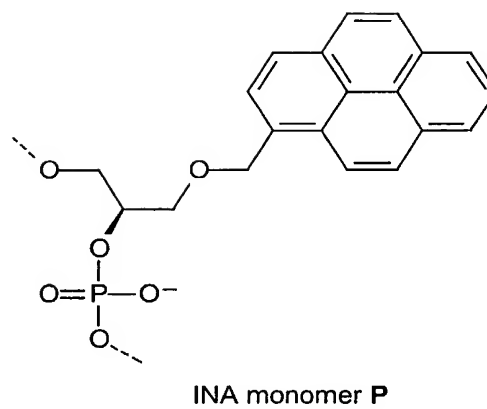
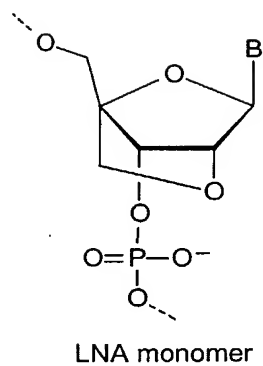
Fig. 29**22/32****Fig. 30**

Fig. 31



23/32

Fig. 32



24/32

Fig. 33

Table 1.

Targets

I

5'-TGT GAT ATG CT

II

5'-TGT GAT^L ATG CT

III

5'-TGT^L GAT^L AT^LG CT

| | | Thermal meltings with target I-III (°C) | | | | | |
|-------|---------------------|---|--------------|-------|--------------|-------|--------------|
| Entry | Probes | I | | II | | III | |
| | | T_m | ΔT_m | T_m | ΔT_m | T_m | ΔT_m |
| 1 | 3'-ACA CTA TAC GA | 42.4 | — | 47.4 | — | 56.7 | — |
| 2 | 3'-ACA CTPA TAC GA | 47.6 | 5.2 | 44.8 | -2.6 | 52.9 | -3.8 |
| 3 | 3'-ACA CTAP TAC GA | 52.5 | 10.1 | 46.5 | -0.9 | 54.5 | -2.2 |
| 4 | 3'-ACA CPTA TAC GA | 44.4 | 2.0 | 47.8 | 0.4 | 57.8 | 1.1 |
| 5 | 3'-ACA CTA TPAC GA | 48.8 | 6.4 | 52.0 | 4.6 | 55.2 | -1.5 |
| 6 | 3'-ACAP CTA TAC GA | 50.6 | 8.2 | 54.2 | 6.8 | 55.1 | -1.6 |
| 7 | 3'-ACA CTA TAPC GA | 50.3 | 7.9 | 54.8 | 7.4 | 53.1 | -3.6 |
| 8 | 3'-ACPA CTA TAC GA | 45.0 | 2.6 | 50.3 | 2.9 | 53.7 | -3.0 |
| 9 | 3'-ACA CTA TAC PGA | 44.4 | 2.0 | 49.2 | 1.8 | 57.2 | 0.5 |
| 10 | 3'-APCA CTA TAC GA | 47.5 | 5.1 | 52.0 | 4.8 | 63.5 | 6.8 |
| 11 | 3'-ACA CTA TAC GPA | 49.6 | 7.2 | 54.3 | 6.9 | 62.7 | 6.0 |
| 12 | 3'-ACA CTA TAC GAP | 48.7 | 6.3 | 53.8 | 6.4 | 60.4 | 3.7 |
| 13 | 3'-ACAP CTA TPAC GA | 54.8 | 12.4 | 60.0 | 12.6 | 52.9 | -3.8 |

25/32

Fig. 34

Table 2.

Probes

T₄-DNA 5'-GATAA TTTT TTATC
 T₄-LNA 5'-GAT^LAA TTTT T^LT^LAT^L MeC^L
 P²-LNA 5'-GPAT^LAA TTTT T^LT^LAT^L MeC^L
 P⁵-LNA 5'-GAT^LAPA TTTT T^LT^LAT^L MeC^L
 P-P-LNA 5'-GPAT^LAPA TTTT T^LT^LAT^L MeC^L

Targets

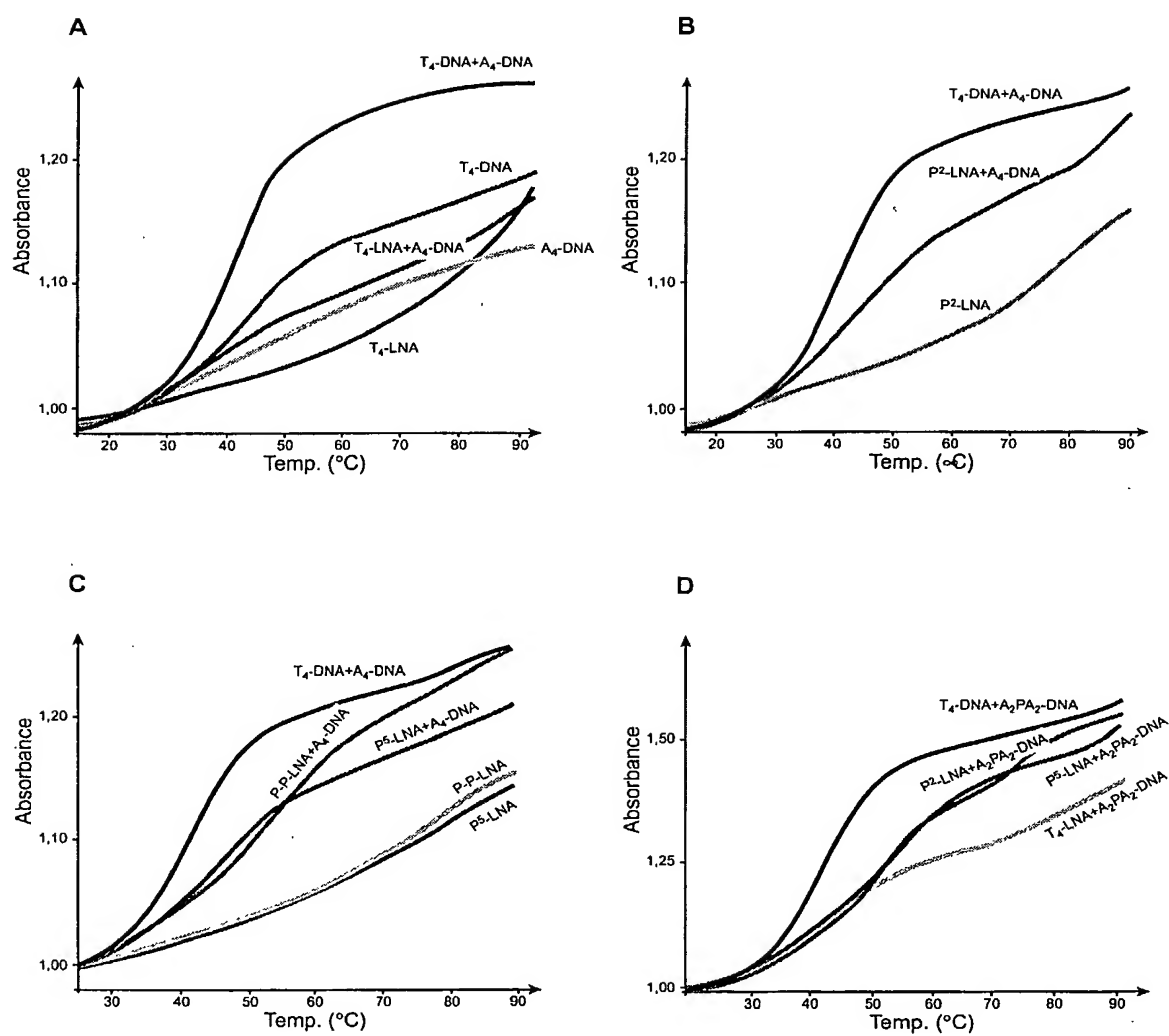
A₄-DNA 3'-CTATT AAAA AATAG
 A₂PA₂-DNA 3'-CTATT AAPAA AATAG
 (APA)₂-DNA 3'-CTATT APAAPA AATAG

| Probes | Targets | | | |
|---------------------|----------------|---------------------|-------------------------------------|-------------------------|
| | Without target | A ₄ -DNA | A ₂ PA ₂ -DNA | (APA) ₂ -DNA |
| Without probe | – | <20 | <20 | <20 |
| T ₄ -DNA | 37.2 | 37.2 | 40.3 | NT |
| T ₄ -LNA | >80 | 37.3 | 44.7 | 42.0 |
| P ² -LNA | (81.1) | 46.4 | 49.9 | NT |
| P ⁵ -LNA | (71.4) | 43.3 | 51.9 | NT |
| P-P-LNA | (69.1) | 53.6 | 61.8 | 83.3 |

ND, not determined.

26/32

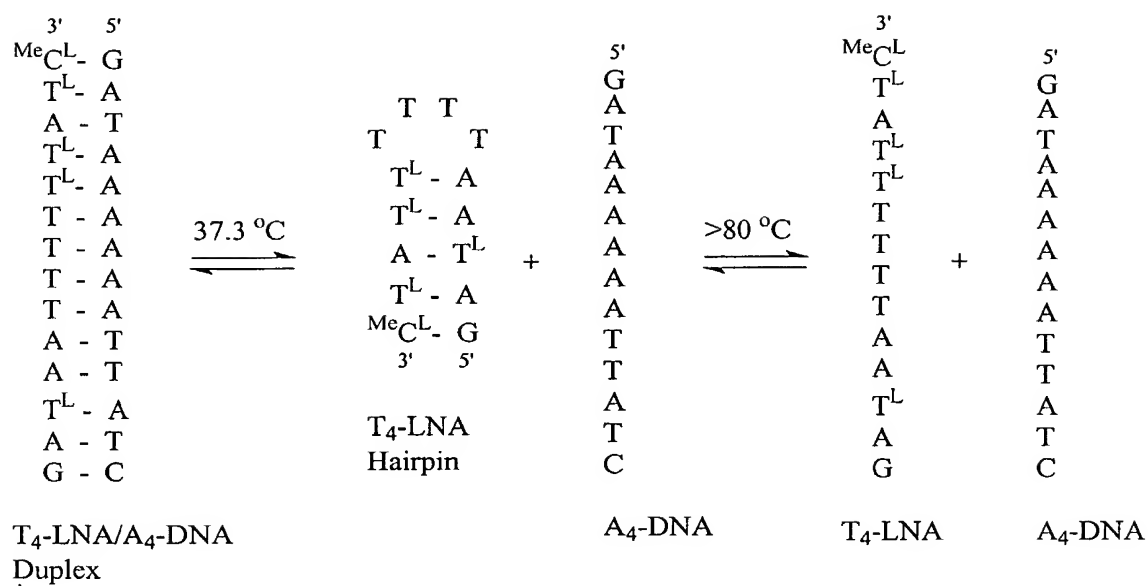
Fig. 35



27/32

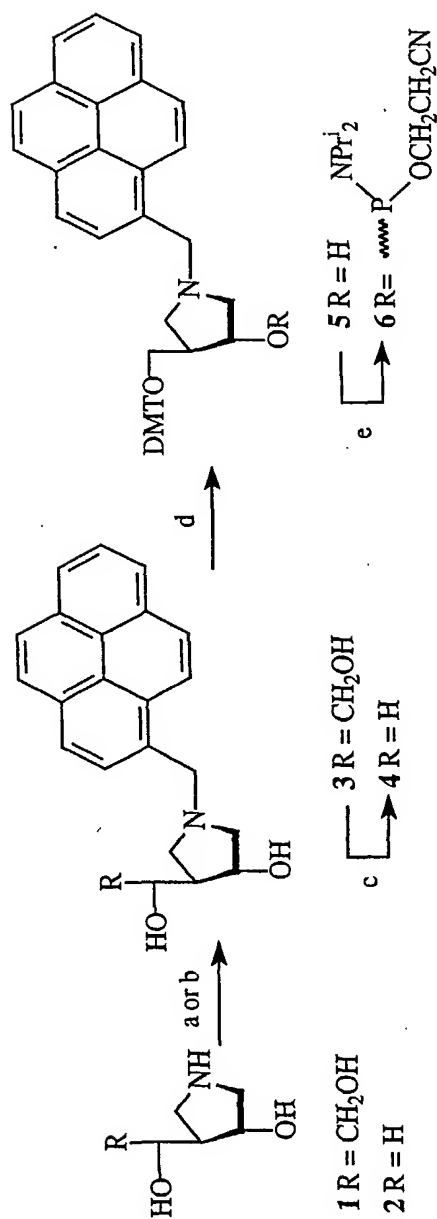
Fig. 36

Scheme 1



28/32

Fig. 37

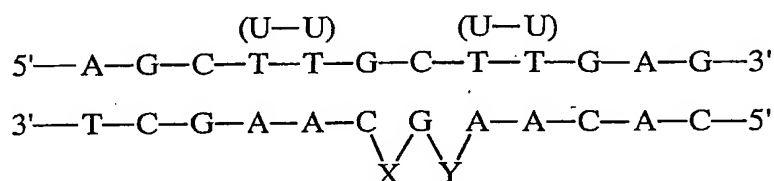


Scheme 1 (a) 1-(chloromethyl)pyrene, Et_3N , DMF; (b) 1-pyrenecarbaldehyde, $NaCNBH_3$, DMF/EtOH (3:1); (c) 1) $NaIO_4$, 2) $NaBH_4$; (d) DMTCl, pyridine; (e) $NC(CH_2)_2OP(NPr)_2$, N,N -diisopropylammonium tetrazolide, CH_2Cl_2 .

29/32

Fig. 38

Sequences and Hybridization Data of Synthesised ODNs in DNA/DNA(RNA) Duplexes

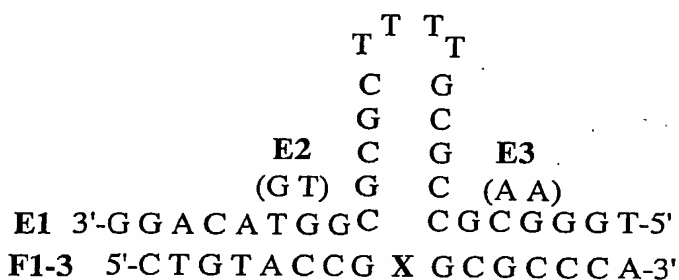


| No | X | Y | T _m (°C) (DNA/DNA) | ΔT _m (°C) | T _m (°C) (DNA/RNA) | ΔT _m (°C) | ΔΔT _m DNA/RNA |
|----|---|---|----------------------------------|----------------------|----------------------------------|----------------------|--------------------------|
| A | — | — | 43.0 | | 42.2 | | |
| B | G | — | 32.2 | -10.8 | 32.6 | -9.6 | 1.2 |
| C | I | — | 41.8 | -1.2 | 32.2 | -10.0 | 8.8 |
| D | I | I | 39.4 | -1.2 | 21.8 | -10.2 | 9.0 |

I = inserted nucleoside analogue 4, ΔT_m = decrease in T_m per modification, ΔΔT_m DNA/RNA = discrimination in T_m between DNA/DNA and DNA/RNA duplexes per modification

Fig. 39

Hybridization Data (T_m °C) for the DNA Three-Way Junction

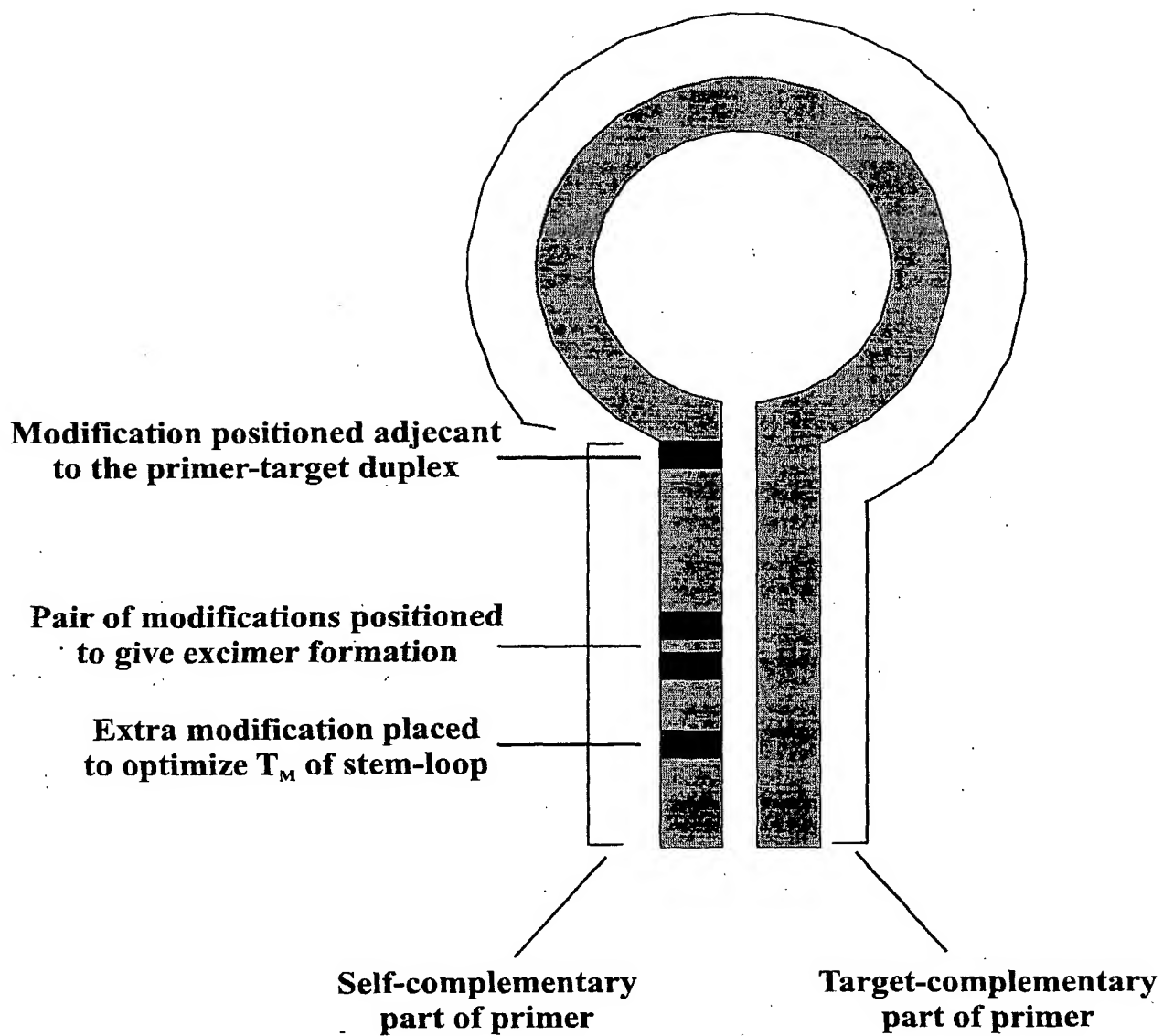


| | F1 (X = 0) | F2 (X = A) | F3 (X = I) |
|----|---------------|---------------|---------------|
| E1 | 38.6 | 39.4 | 48.6 |
| E2 | <18.0 | 20.2 | 24.2 |
| E3 | <18.0 | 19.4 | <18.0 |

I = inserted nucleoside analogue 4

30/32

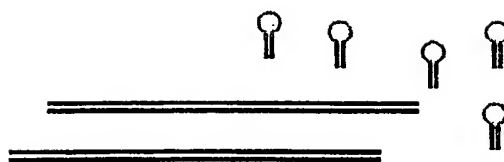
Fig. 40



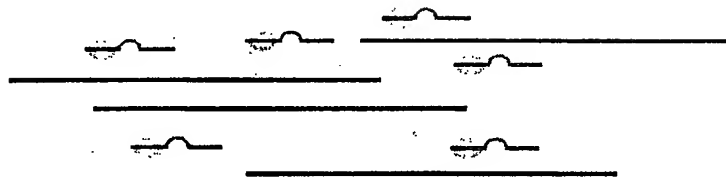
31/32

Fig. 41

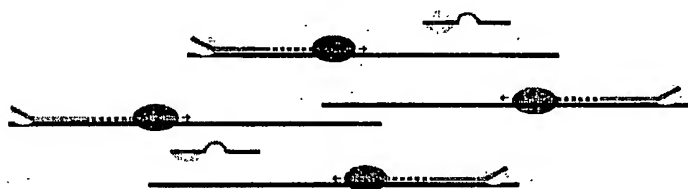
A



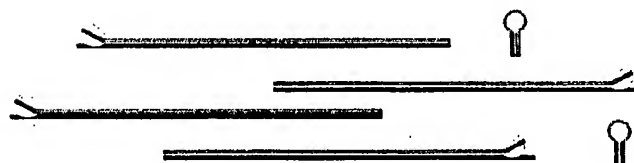
B



C



D



Beacon-design primer



Target-complementary part of oligo



Target DNA

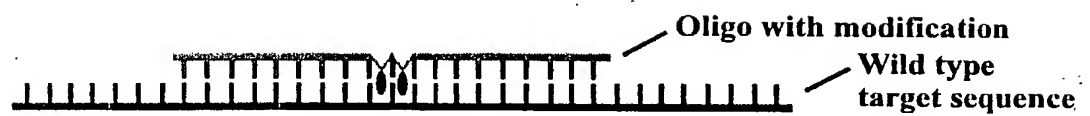





Fluorescent oligo modifications



Amplified DNA

32/32

Fig. 42**A Complete complementarity****B Mismatch and excimer-formation**

-  Fluorescent modification
-  Point mutation mismatch
-  wt base pairing